



Enzyme discovery for brown seaweed fucoidan modification

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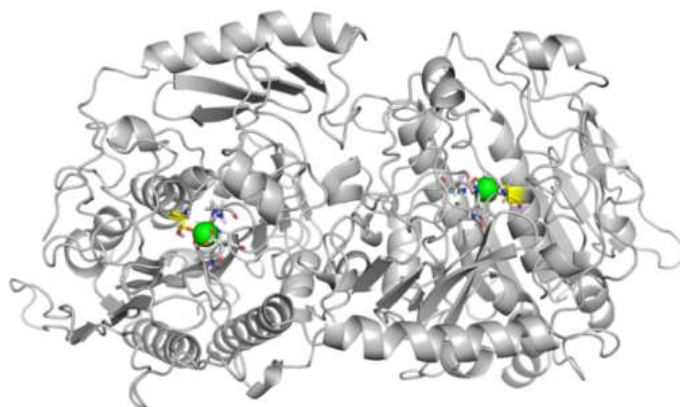
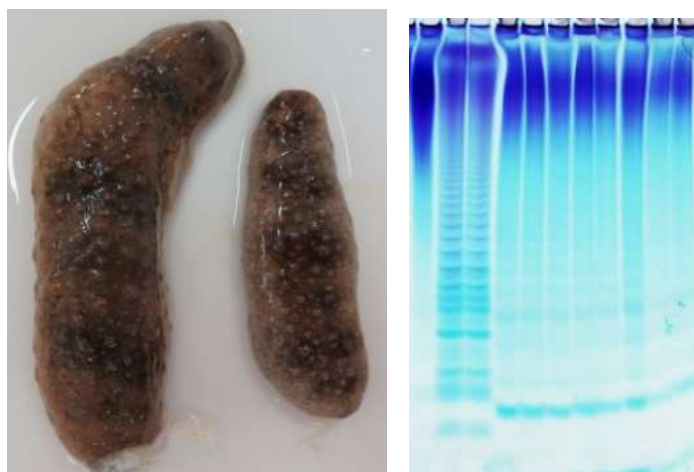
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Enzyme discovery for brown seaweed fucoidan modification



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Ph.D. Thesis

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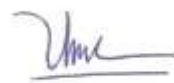


Preface

This thesis is submitted as one of the requirements for obtaining the PhD degree at the Technical University of Denmark and comprises the research carried out at the Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, at the Technical University of Denmark and at the Nhatrang Institute of Technology Research and Application from January 2015 to February 2018.

The work was carefully supervised by Professor Anne S. Meyer and co-supervised by first Prof. Jørn D Mikkelsen, Mateusz Lezyk and later Maria Dalgaard Mikkelsen, all from the Centre for Bioprocess Engineering. The project was also supervised by Associate Professor Tran Thi Thanh Van from the Nhatrang Institute of Technology Research and Application, Vietnam.

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Hang Thi Thuy Cao

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Abstract

The objective of this PhD study was to discover enzymes (fucoidanases and sulfatases) that are able to modify fucoidan from the brown seaweed *Sargassum mcclurei*.

In this work, the discovery and characterization is reported of novel enzymes of marine bacteria isolated from sea cucumber gut. A collection of marine bacteria from sea cucumber gut in the Vietnam Sea was created and used for screening for ability to modify fucoidan from *Sargassum mcclurei* and *Turbinaria ornata*. Strains with fucoidan modifying potential were subsequently identified by 16S RNA analyses.

The marine bacterial strains *Pseudoalteromonas* MB47 and *Cobetia* MB87 were selected for further study and their genome sequences were analyzed. The *Pseudoalteromonas* MB47 genome contained six potential sulfatases. One of these sulfatases, Ps1595, was found to be active on synthetic substrates (p-nitro catechol sulfate and p-nitro phenyl) and interestingly, for the first time, on fucoidan from *S. mcclurei*. The properties of the Ps1595 sulfatase were characterized on p-NCS substrate. The optimal reaction conditions for Ps1595 sulfatase were pH 6.5, 68°C, 10mM CaCl₂. The K_m and V_{max} were 0.95 ± 0.15 and 26.6 ± 1.2 U, respectively, for the reaction without NaCl. With 125mM NaCl in reaction mixture, the K_m did not change but V_{max} decreased by 25%.

No fucoidanase could be determined from the sequence data of *Pseudoalteromonas* MB47 and *Cobetia* MB87, therefore we used online databases to select fucoidanases that might be able to degrade fucoidan *S. mcclurei*. We selected five microbial fucoidan degrading enzymes, including three endo-fucoidanases FcnA2, Fda1 and Fda2 (EC3.2.1.-, GH 107) and two unclassified endo-fucoglucuronomannan lyases, FdlA and FdlB. The five fucoidanases were expressed heterologously in *E. coli* and purified. These enzymes were used to treat various fucoidan substrates and the resulting oligosaccharide product profiles were assessed by carbohydrate-polyacrylamide gel electrophoresis (C-PAGE) and size exclusion chromatography. For the first time we show that all five fucoidanases in this study can degrade *S. mcclurei* galactofucan-fucoidan, but the recombinant FcnA2, Fda1 and Fda2 enzymes were unstable to different degrees. However, active and more stable enzymes were obtained by truncation of the C-terminal end (by removal of up to 47% of the protein in Fda1).

In conclusion, the data obtained have implications for use of these enzymes, including the stabilized versions, in fucoidan processing. Hopefully, this will result in manufacturing of homogenous bioactive fucoidan oligomer products for use in the medicines industry.

Dansk sammenfatning

Formålet med dette ph.d.-studie var at finde nye enzymer (fucoidanaser og sulfataser), der er i stand til at modificere fucoidan fra den brune tang *Sargassum mcclurei*. Som del af dette studie, er der fundet og karakteriseret nye enzymer fra marine bakterier, der er isoleret fra maverne fra søpølser fra det vietnamesiske hav. Disse marine bakterier er blevet isoleret og screenet for deres evne til at modificere fucoidan fra *Sargassum mcclurei* og *Turbinaria ornata*. Bakterierne med fucoidan modificerende egenskaber, blev efterfølgende identificeret ved hjælp af 16S RNA analyser. To af disse blev endvidere udvalgt, *Pseudoalteromonas* MB47 og *Cobetia* MB87, og genom-sekventeret. *Pseudoalteromonas* MB47 genomet indeholdte seks mulige sulfataser, identificeret gennem sekvens alignments med andre kendte sulfataser. Disse sulfataser blev udtrykt heterologt i *E. coli* og én af sulfataserne, Ps1595, viste sig at være aktiv på de syntetiske substrater 4-nitrocatecholsulfat (NCS) og para-nitrophenyl-sulfat (pNPS). Egenskaberne af Ps1595-sulfatasen blev undersøgt på substratet NCS. De optimale reaktionsbetingelser for Ps1595-sulfatasen var: pH 6,5, 68 °C og 10 mM CaCl₂. K_m og V_{max} var henholdsvis 0,95 ± 0,15 og 26,6 ± 1,2 U for reaktionen uden NaCl. Med 125 mM NaCl i reaktionsblandingen ændredes K_m ikke, men V_{max} reduceredes med 25 %. Endvidere var Ps1595 også i stand til at desulfatere oligosakkarider fra fucoidan fra *S. mcclurei* og er herved den første karakteriserede fucoidan aktive sulfatase.

Sekvensdataene fra *Pseudoalteromonas* MB47 og *Cobetia* MB87 indeholdte ikke nogen sekvenser, der lignede andre kendte fucoidanaser, derfor benyttede vi som alternativ, online-databaser til at udvælge forskellige fucoidanaser, der muligvis ville kunne nedbryde fucoidan fra *S. mcclurei*. Vi udvalgte fem mikrobielle fucoidanaser inklusiv tre endo-fucoidanaser (EC3.2.1.-, GH 107): FcnA2, Fda1, Fda2 og to endo-fucoglucuronomannan lyaser: FdlA og FdlB. De fem fucoidanaser blev udtrykt heterologt i *E. coli* og oprenset. Enzymatisk behandling af fucoidan-substrater resulterede i oligo-sakkarid produkter, der blev undersøgt vha. kulhydrat-polyacrylamid gelelektroforese (C-PAGE) og størrelses-kromatografi. Alle fucoidanaserne var i stand til at nedbryde fucoidan fra *S. mcclurei* og det er herved første gang enzymer er identificeret, der kan nedbryde det komplekse *S. mcclurei* galactofucan-fucoidan. De rekombinante FcnA2-, Fda1- og Fda2-enzymmer var ustabile i forskellige omfang. Imidlertid blev aktive og mere stabile enzymer opnået ved trunkering af den C-terminale ende (ved fjernelse af op til 47 % af proteinet i Fda1).

De opnåede resultater fra dette studie vil have stor indflydelse på processeringen af fucoidan i fremtiden, især stabiliseringen af fucoidanase enzymerne. Forhåbentlig vil det i fremtiden

resultere i fremstillingen af homogene bioaktive fucoidan oligo-sakkarider, der kan bruges i medicinalindustrien.

List of Publication**I. Isolation and screening of aerobic marine bacteria from sea cucumber to identify novel fucoidan modifying enzymes**

Hang Thi Thuy Cao, Maria Dalgaard Mikkelsen, Mateusz Jakub Lezyk, Nanna Rhein-Knudsen, Bui Minh Ly, Van Thi Thanh Tran, Thuan Thi Nguyen, Thinh Duc Pham, and Anne S. Meyer.

(Will be submitted to Enzyme and Microbial Technology)

II. A thermostable fucoidan active sulfatase (co-first author)

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III. Novel enzyme actions for sulfated galactofucan depolymerisation and a new engineering strategy for molecular stabilization of fucoidan degrading enzymes

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List of Abbreviations

2-AMAC	2-aminoacridone
ANTS	8-aminonaphthalene-1,3,6-trisulfonate sodium
AGCs	Analysis of the hydrophobic clusters
AnSME	anaerobic sulfatase-maturing enzyme
BSA	Bovine serum albumin
BLAST	Basic Local Alignment Search Tool
FGH	Catalytic nucleophile as
CAZy	Carbohydrate-Active enZYmes Database
FGly	Formylglycine
DEAE	Diethylaminoethyl cellulose
ESI	Electrospray ionization
FCSPs	Fucose-containing sulfated polysaccharides
GAG	Glycosaminoglycans
Cetavlon	Hexadecyltrimethylammonium bromide
HIV	The human immunodeficiency virus
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IR-spectroscopy	Infrared spectroscopy
MALDI	Matrix Assisted Laser Desorption/Ionization
MB	Marine bacterial medium
pNCS	p- nitro catechol sulfate
pNPS	p-nitro phenyl sulfate
C-PAGE	Carbohydrate - Polyacrylamide gel electrophoresis
PSI-BLAST	Position-Specific Iterated BLAST
SFGM	Sulfated fuco-glucuronomannan
SEC	Size exclusion chromatography
FACE	Fluorophore-assisted carbohydrate electrophoresis
X-Fuc	5-bromo-4-chloro-3-indolyl- α -L fucopyranose
X-SO ₄	5-Bromo-4-chloro-3-indolyl sulfate potassium salt

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Thesis motivation and outline

Seaweeds are rich in polysaccharides that differ from terrestrial plant polysaccharides both with respect to the relative abundance of specific building blocks and notably with respect to the type of bonds that tie the monomeric units together. Brown algae in particular contain several compounds with biological activity, such as polysaccharides, iodine organic products, mannitol, macro- and microelements, vitamins, unsaturated fatty acids, and other biogenic compounds (Deeniaud et al., 2014). Among these compounds, fucoidans from brown seaweeds have been reported to possess many biological activities such as immuno-modulatory effects, antitumoural, anticoagulant and antiviral activity, including activity against HIV, herpes and hepatitis viruses (Fitton et al., 2015). All these properties make fucoidans promising for medicine, the food industry and agriculture. However, drugs based on them have not yet been created. A basic tenet behind this thesis is that production of uniform, homogenous, fucoidan oligomer preparations would provide an improved basis for assessing structure-function relations and in turn for employing fucoidans in functional medical formulations and/or as functional food ingredients. Due to the selectivity and capacity of enzymes to act under mild conditions, enzymes have most promise as a way to standardize natural polysaccharides; enzymes could be used to obtain preparations of uniform molecular size, establish their biological activity and structure-function relations, and reduce side effects, and hence potentially be used to tailor-make specific fucoidan structures by targeted bio catalysis. However, only very few enzymes have been described that can modify and/or degrade native fucoidans. There is very little knowledge available on substrate attack preferences and optimal reactions conditions, and on the robustness of fucoidan modifying enzymes.

Thus, this PhD study has focused on providing the enzymes for attaining controlled preparation of uniform, well-defined fucoidan preparations via discovery of fucoidan modifying enzymes (fucoidanases and sulfatases) with the capability to modify fucoidans in different ways. The objects of our research are fucoidanases available online and bacteria that participate in catabolism of fucoidans in sea cucumber guts. The basis for the PhD was shaped by the following general hypotheses.

1. *Sargassum mcclurei* and *Turbinaria ornata* are brown seaweeds commonly distributed in the Vietnam Sea. Sea cucumbers, which are among the algal feeders in marine ecosystems, contain a gut microbial population with potential for producing

polysaccharide-degrading enzymes, including fucoidanases and sulfatases. Hence, the gut microbial population of sea cucumbers represents a pool of potential fucoidan modifying enzymes, including endo- and exo-fucoidanases, and sulfatases, and is a very interesting subject to study.

2. Natural fucoidans are sulfated, i.e. they carry sulfate substitutions directly on the backbone fucosyl residues. A basic assumption is that the sulfate groups (and the sulfation pattern) on fucoidan may affect the bioactivity of fucoidan. The sulfate groups may furthermore affect the activity of the fucoidanases either by sterically blocking access of the fucoidanases to the backbone structure and bonds or by being necessary for the fucoidanases to catalyze the cleavage of the glycosidic bonds. However, the fucoidan sulfatases have been very poorly described until now and none of them have been characterized, and in particular not on native fucoidans. Hence, based on the hypothesis that sulfatase action may be a requirement for proper endo-fucoidanase action, discovery and characterization of fucoidan sulfatases was included in the thesis work. The assessment of sulfatase action on different fucoidans may also lead to knowledge about the function of the sulfates in fucoidan.
3. Fucoidan from *S. mcclurei* is a special type of sulfated galacto-fucan of particular interest in Vietnam because *S. mcclurei* is a prevalent type of brown seaweed in Vietnam. Moreover, the *S. mcclurei* galacto-fucan has been found to have anti-cancer and anti-viral properties and the overall structure has been resolved at Nhatrang institute of Technology Research and Application, Vietnam. Prior to this work, no fucoidanases had been reported to act on *S. mcclurei* fucoidan. A particular objective was therefore to identify enzymes capable of modifying *S. mcclurei* fucoidan, based on the hypothesis that such enzymes can be used to prepare bioactive oligosaccharides from *S. mcclurei*. Thus, based on available knowledge of this structural fucoidan, we tried to find potential enzymes from the online database to modify this substrate. There are currently about 11 fucoidanases in the database which have not yet been characterized completely with respect to their biochemical properties as well as substrate specificities. Therefore in this study, fucoidans with different structures were also used as substrate for the fucoidanase enzymes. The results will expand our theoretical knowledge of the structural and functional properties of these enzymes and allow them to be used to identify the structure of natural polysaccharides.

4. With our knowledge about fucoidanases, native fucoidanases are highly prone to degradation and subsequent loss of activity during the isolation and purification processes. This is likely the cause of the low number of fucoidanases characterized to date, even though the first fucoidanase was found several decades ago. Therefore, in this study, we wanted to identify the optimal reaction conditions of fucoidanases and find out how to stabilize these enzymes.

To address the different hypotheses, the following objectives were defined:

1. Discovery of novel fucoidanases and sulfatases by isolation and screening bacteria from the gut of sea cucumbers.
2. Characterization of fucoidan sulfatases.
3. Selection of fucoidanases in online databases.
4. Production and expression of the fucoidanases in a production system that would assure high protein yield and activity.
5. Use of recombinant enzymes to degrade selected fucoidans and analysis of the products.
6. Stabilization of fucoidanases by molecular engineering.

The work performed during this PhD study, founded on the objectives and hypotheses mentioned above, is described in the following chapters of the thesis. **Chapter 1** introduces the theory of fucoidan and fucoidan modifying enzymes. **Chapter 2** describes the isolation and screening of aerobic marine bacteria from the gut of sea cucumbers in order to produce fucoidan modifying enzymes. **Chapter 3** describes the fucoidan sulfatases: cloning, expression and biochemical characterization. **Chapter 4** focuses on the identification, expression and purification of fucoidanases for degrading different fucoidan substrates. **Chapter 5** describes a new engineering strategy for molecular stabilization of fucoidan degrading enzymes. **Chapter 6** includes the conclusions that we draw from this PhD study and future perspectives.

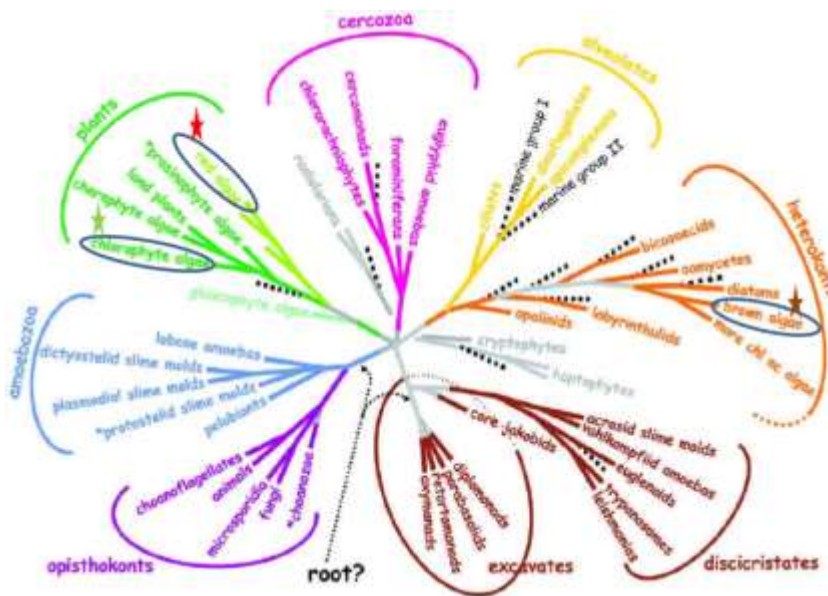


Figure 1.1 Eukaryotic phylogeny. Macroalgae are phylogenetically distant, with red and green algae in the extended kingdom of plants, while brown algae are in the heterokonts (Baldauf, 2003).

include the largest of all algae. They are also the group of macroalgae for which the largest number of studies concerning biological activities is available. There is no doubt that the large size of many brown seaweeds has made them very suitable subjects to test for biological activities and also facilitates extraction of large amounts of the associated bioactive compounds.

Vietnam has a coastline of 3,200 km with a warm and humid monsoon tropical climate, favorable for seaweed growth. Survey results showed that Vietnam has extensive seaweed resources, and about 800 species of seaweed have been identified (Fig 1.2). Among them, many genera have a high natural production, such as *Sargassum* and *Turbinaria*, which generate about 20,000 dry tons in nature annually (Ly and Hau, 2010). Thus the potential of Vietnam brown seaweed biomass production is really large.

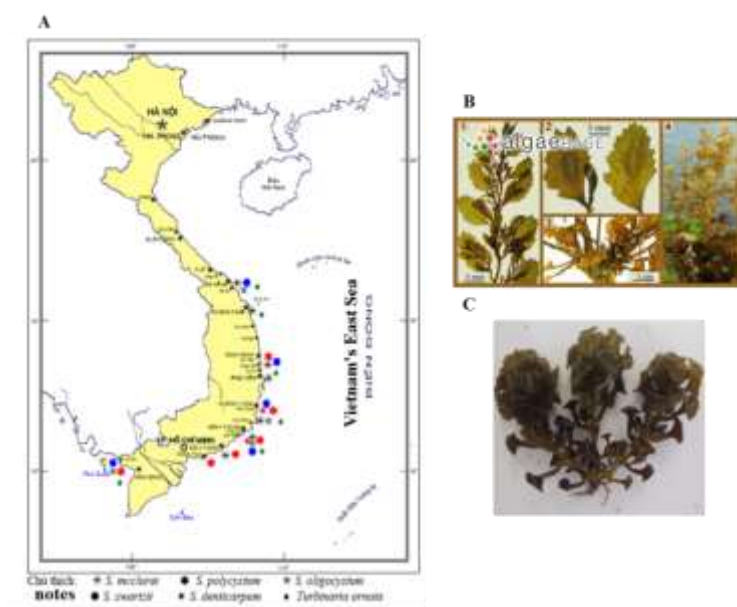


Figure 1.2 Brown seaweed in Vietnam. A) The distribution of brown seaweed in the Vietnam Sea. B) *Sargassum mcclurei*, Vietnam (Source: www.algaebase.org). C) *Turbinaria ornata* in Nha Trang Bay, Vietnam.

Brown algae are typically used for the production of alginate, which is commercially used as an ingredient for different industrial, biotechnology and food applications. Fucose sulfated polysaccharides, notably fucoidan, are known to exhibit many bioactive properties.

1.2. Fucoidan: Sulfated polysaccharides from the sea with complex structure

Fucoidans or fucose-containing sulfated polysaccharides (FCSPs) are marine sulfated polysaccharides that have not yet been found in any terrestrial organisms. They essentially contain fucose and sulfate groups with some other groups, such as galactose, xylose, mannose and uronic acids. Fucoidan is made up of α -L-fucose units linked by $\alpha(1\rightarrow4)$ and/or $\alpha(1\rightarrow3)$

glycosidic bonds and sulfated at positions 2 and/or 3 and/or 4 (Ale & Meyer, 2013). Fucoidans are produced by brown marine macroalgae (seaweed) and certain marine invertebrates, such as sea cucumbers and sea urchins. To date, naturally occurring fucans without sulfate groups have never been reported.

Fucoidan was first described by Vasseur (1948) from a marine invertebrate. He extracted a sulfated methyl-pentose containing polysaccharide from the eggs of a sea urchin (Vasseur et al. 1948). Since then, sulfated fucans have been isolated from the egg jelly coat of many species of sea urchins, such as *Strongylocentrotus droebachiensis*, *Echinus esculentus*, *Psammechinus miliaris*, *Echinocardium cordatum*, *Brissopsis lyrifera* (Alves et al., 1999) and from the body wall of another type of marine echinoderm, the sea cucumber, for example *Acaudina Molpadioides* (Yu et al., 2014).

Fucoidans have been found in most brown seaweeds, as Usov and Bilan (2009) reported in their review (Usov and Bilan, 2009). Fucoidan polysaccharides were extracted for the first time 100 years ago, in 1913, from various brown seaweeds. Already then, Kylin (1913) reported that extracted fucoidans mainly consisted of fucose. Fucoidans are one of the components of the primary cell walls of brown seaweeds (Popper et al., 2011). In 2014, Deniaud-Bouët et al. described the comprehensive analysis of the cell wall composition of five species of Fucales; they found that brown algae cell walls contain alginate, cellulose, phlorotannins, proteins and iodide (Deniaud et al. 2014) (Fig.1.3).

The content of fucoidans in brown algae depends on the species and on the stage of development of the algae, and may vary from 0.1 to 20% of the dry weight, with the most common content being around 2-10% (Zvyagintseva et al., 2003).

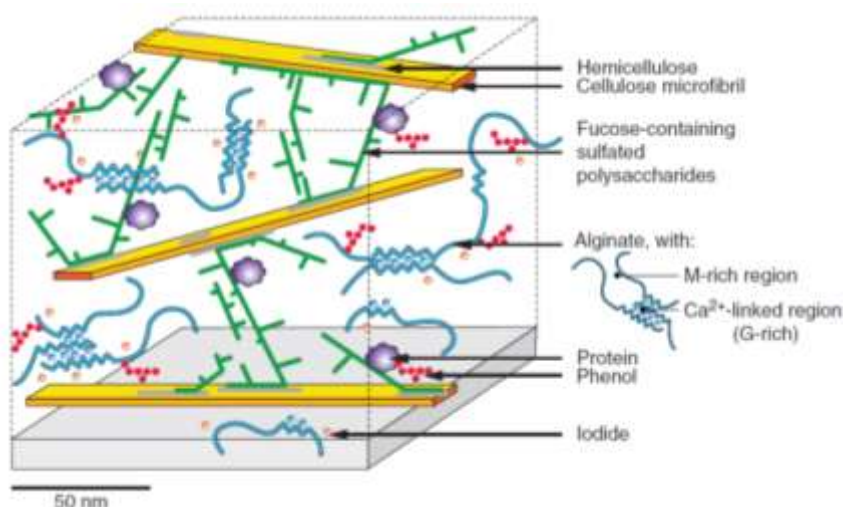


Figure 1.3 Cell wall model of a brown alga from the order Fucales (Deniaud et al., 2014)

Little is known about the function of fucoidan in marine organisms. However, in sea urchins, the sulfated fucans were found in a gelatinous layer surrounding the eggs, where it was found to play a role in induction of the acrosome reaction during sea urchin fertilization (Mourão, 2007). The role of sulfated fucans in the body wall of sea cucumbers is less understood, but an involvement in the maintenance of the integrity of the body wall has been suggested (Mourão and Bastos, 1987). In algae, some studies have shown a correlation between fucoidan content and the depths at which brown seaweeds grow; the closer to the surface, the higher the fucoidan content (Evans, 1989). Furthermore, fucoidans appear to play a role in algal cell wall organization (Kloareg & Quatrano, 1988) and could be involved in the cross-linkage of alginate and cellulose (Mabeau et al., 1990). Deniaud-Bouët et al. also reported that fucoidans interlock the cellulosic scaffold while the alginate–phenol linkages are key players in regulating the rigidity of the wall (Deniaud et al. 2014). A role for fucoidans in the morphogenesis of algae embryos has also been suggested (Bisgrove & Kropf, 2001).

Although the content of fucoidans in brown algae is not so high, fucoidans have attracted attention for a long time due to diverse biological activities, low toxicity, and plant origin (Berteau & Mulloy, 2003).

1.2. Biological activities of fucoidan and application

Fucoidans have been reported to possess various biological effects *in vitro* and *in vivo* such as anti-inflammatory, anticoagulant, antithrombotic (Kusaykin et al., 2008) (Cumashi et al., 2007), antiviral including anti-HIV (Lee et al., 2004), (Thuy et al., 2015), immunomodulatory (Raghavendran et al., 2011), antioxidant (Jin et al., 2008), and antitumor activity (Zhuang et al., 1995), (Alekseyenko et al., 2007). Fucoidans from *S. mcclurei* and *Turbinaria ornata* seaweeds isolated from the Vietnam sea were reported to have antiviral and anticancer activities (Pham et al., 2013), (Thuy et al., 2015). These fucoidans have also been used as a functional food in Vietnam, such as in Fucogastro products (<https://fucoidan.com.vn>). Using anion chromatography Macro-Prep DEAE, *S. mcclurei* fucoidan was separated into three fractions of different monosaccharide composition and different sulfate content. All fucoidan fractions were reported to exhibit colony formation inhibition in colon cancer DLD-1 cells (Pham et al., 2013). Therefore these fucoidan fractions are potential antitumor agents. Fucoidan from *T. ornata* contained two fractions, ToF1 and ToF2, when separated by anion exchange chromatography Macro-Prep DEAE. The anticancer effect of fucoidans ToF1 and ToF2 was investigated by the soft agar colony formation assay using human colorectal HT-29, breast T-47D adenocarcinoma and malignant melanoma SK-MEL-28 cell lines (Ermakova et al. 2015b).

The activities of *Fucus evanescens* fucoidan were reported by Rosa V. Menshova and summarized in her review from 2016. The fucoidan was shown to have antiviral, anticoagulant, anticancer, antioxidant and immunomodulatory activities. At the G.B. Elyakov Pacific Institute of Bioorganic Chemistry of the Far Eastern Branch of the Russian Academy of Sciences, fucoidan from *F. evanescens* was used to create the first Russian food supplement based on fucoidan: “Fucolam”. “Fucolam” possess all of the positive biological properties that have been established for fucoidan from *F. evanescens* (Menshova et al., 2016).

The bioactivity of fucoidan from *Fucus vesiculosus* is the most studied because it was the first commercial fucoidan. Fucoidan from *F. vesiculosus* was reported to show anticoagulant activity *in vitro* and *in vivo* (Bernardi & Springer, 1962) and antithrombic activity (Church et al., 1989). Fucoidan was also reported to mediate a variety of significant biological effects, such as blocking sperm–egg binding in diverse species, blocking infection of human cell lines with HIV, herpes and cytomegalovirus, blocking cell–cell binding mediated by L-selectin recognition of oligosaccharides and other molecular mechanisms by interfering with cell-to-cell recognition (Patankar et al., 1993). Crude extracts of fucoidan from *F. vesiculosus* were also demonstrated to induce intraperitoneally induced apoptosis of 4T1 breast cancer cells in tumor-bearing mice, but the pure fucoidan did not cause apoptosis of some other cancer cells *in vitro* (Negishi et al., 2013). *F. vesiculosus* fucoidan has been found to have immunostimulatory effects on various types of immune cells, including macrophages and dendritic cells (Kim et al., 2015).

The bioactivity of fucoidan from brown algae are known to be dependent on several structural parameters, such as the degree of sulfation and acetylation, the monosaccharide composition, type of glycosidic bonds, and others (Ale et al., 2011), (Soeda et al., 2000). A relationship between sulfate content and the anticoagulant activities has been reported (Takashi & Nagumo, 1991). Fucoidan from *Ascophyllum nodosum* are mainly sulfated at C2, to a lesser extent at C3, and some at C2 and C3. This fucoidan was the first to be reported to have anticoagulant activity and this anticoagulant activity was related not only to molecular weight and sulfate content but also to the levels of sulfated at C2 and C2, C3 (Chevolot et al., 1999). Another author reported that the sulfate content of fucoidan is one of the most important factors for its anticoagulant effects (Qiu et al. 2006). Fucoidan from *F. vesiculosus* (Sigma) was chemically sulfated by using chlorosulfonic acid–pyridine complex. The sulfated compound exhibited four times higher anticoagulant activity in doubling prothrombin time of normal citrated human plasma in comparison to native fucoidan (Qiu et al., 2006). The desulfated fucoidan from *T. ornata* exhibited slight activity against colony formation in SK-MEL-28 cells and did not inhibit colony

formation in HT-29 and T-47D cells. While the native *T. ornata* fucoidan inhibited colony formation of SK-MEL-28, HT-29, T-47D cells by 44%, 24%, and 15%, respectively (Ermakova et al. 2015b).

The specific biological activities of fucoidans are also associated with their structures. The formation and growth of colonies of breast cancer cells were suppressed by galactofucans from *Saccharina japonica* and *Undaria pinnatifida* (Vishchuk et al. 2011). Human colon cancer cells were more sensitive to fucoidan from *Saccharina cichorioides* (consisting of (1→3)- α -L-fucose residues) while human melanoma cells were more sensitive to fucoidan from *Fucus evanescens* (Vishchuk et al. 2013b).

Although fucoidan have potential as biologically active compounds, the high molecular mass (from 13kDa for *Ascophyllum nodosum* fucoidan (Daniel et al., 2001) to 950 kDa for *Hizikia fusiforme* fucoidan (Li et al., 2006) and viscous nature of fucoidan have hampered their applications, especially as therapeutic agents. Chemical, radical, or enzymatic methods can be used to obtain bioactive oligosaccharides with low molecular weight (LMW) (Table 1.1).

Table 1.1 Various methods used to prepare low molecular weight fucoidan and their bioactivities

Method	Sources of seaweeds	Bioactivity	Reference
Radical	<i>Ascophyllum nodosum</i>	Antithrombotic	(Chabut et al., 2003)
Radical	<i>A. nodosum</i>	Antithrombotic	(Durand et al., 2008)
Radical	<i>A. nodosum</i>	Anticancer	(Alkhatib et al., 2006)
Radical	<i>A. nodosum</i>	Anticancer	(Hlawaty et al., 2011)
Radical	<i>F. vesiculosus</i>	Anticancer	(Lake et al., 2006)
Acid	<i>Laminaria japonica</i>	Antithrombotic	(Zhu et al., 2010)
Acid	<i>U. pinnatifida</i>	Anti-inflammatory	(Park et al., 2010)
Acid	<i>L. japonica</i>	Antioxidant and anticoagulant	(Wang et al., 2009)
Acid	Sporophyll from <i>U. pinnatifida</i>	Anticancer	(Yang et al., 2008)
Acid	<i>F. vesiculosus</i>	Anticancer	(Azofeifa et al. 2008)
Gamma irradiation	Sigma fucoidan <i>Fucus vesiculosus</i>	Antioxidant	(Choi et al., 2009)
Enzyme	Sporophyll of <i>Undaria pinnatifida</i>	Anticoagulant	(Synytsya et al., 2010) (Kim et al., 2010)
Enzyme	<i>Fucus evanescens</i>	Anticancer	(Silchenko et al. 2017b)
Enzyme	<i>Sargassum horneri</i>	Anticancer	(Silchenko et al. 2017a)

When investigating the biological properties of fucoidans, the most important factor is the purity of the fucoidans. However, uncharacterized crude preparations are often used even in scientific research (JingWanga et al., 2008), (Hu, Liu, Chen, Wu, & Wang, 2010), (Costa et al., 2011). In

brown seaweeds, not only fucoidan but also secondary metabolites such as polyphenols and other UV absorbing compounds have bioactivity (Michailovna et al., 2005). These substances are thought to be strongly associated with fucoidans and removing of them is very difficult. Eliminating these secondary metabolites includes preprocessing the algae with organic solvents (Michailovna et al., 2005).

The highest purity of fucoidans is obtained after separation techniques, such as weak anion exchange chromatography, which separate the different fucoidan populations, eliminate the last low molecular impurities, and result in a more homogenous and whiter sample. Brown seaweeds can synthesize fucoidans of various structures. For example, 16 fractions of fucoidans with different contents of linkages of $\alpha(1\rightarrow3)$ - and $\alpha(1\rightarrow4)$ – L – fucose and degrees of sulfation and acetylation were obtained from crude, commercially available fucoidans from *F. vesiculosus* (Nishino et al., 1994). Different batches of this crude fucoidan differ in color and constitution. The composition of fucoidan also depends on the location of algal harvest, the conditions of the polysaccharide extraction procedure and method of purification. In the next section, methods of extraction and purification will be described.

1.3. Fucoidan Extraction and Analysis

1.3.1. Isolation of fucoidans

Brown seaweed contains alkali-soluble polysaccharides (alginates) and water-soluble polysaccharides (laminaran, fucoidan). Fucoidan can therefore be extracted from algae with water (Adhikari et al., 2006) or dilute acids (Hemingsom et al., 2006), (Yoon et al., 2007) at room or slightly elevated temperatures. In a popular procedure, 2% aqueous solution of calcium chloride is used to convert alginate contained in the biomass to an insoluble calcium salt (Bilan et al., 2002), (Ponce et al., 2003). Low molecular weight components, such as polyphenol, lipid, iodine and pigments, can be preliminarily removed by treating the biomass with organic solvents, for instance, a homogeneous mixture of methanol, chloroform and water (4:2:1), which effectively dissolves both polar and nonpolar substances while leaving biopolymers undissolved. Apart from fucoidans, aqueous extracts of seaweeds also contain polynomic molecules differing in composition and charge, such as laminaran, uronic acid, proteins, polyphenols, etc. Fucoidan can be precipitated from the mixture as an insoluble salt with a cationic detergent, such as trimethylce-tylammonium bromide (cetavlon, cetrimide) (Bilan et al., 2002), (Ivanova et al., 2003). In a number of cases, a stepwise dissolution of the precipitate allows further fractions differing in chemical nature to be obtained (Ponce et al., 2003). However, most often the precipitate is converted to a water-soluble sodium or calcium salt, and

subsequent fucoidan purification and fractionation procedures are carried out by anion-exchange chromatography. The most popular anion exchangers used for these purposes are DEAE-sephadex (Chizhov et al., 1999), DEAE-sephacel (Bilan et al., 2002) and DEAE-sepharose (Mabeau et al., 1990), (Adhikari et al., 2006). The separation process can be controlled by electrophoresis in a thin layer of agarose gel (Silva et al., 2005), (Rocha et al., 2005) or on plates of cellulose acetate (Hemmingson et al., 2006). More or less charge-homogeneous fractions are sometimes additionally purified by gel permeation chromatography (Rocha et al., 2005) and used afterwards for structural analysis.

Fucoidan from *S. mcclurei* (Pham et al., 2013), *T. ornata* (Ermakova et al. 2015b), *F. evanescens*, *L. cichorioides* (Anastyuk et al., 2010) and *U. pinnatifida* (Vishchuk et al. 2013b) were prepared by Patent WO 2005/014657. In this approach, fucoidan was separated from other polysaccharides and then fractionation was conducted in an anion exchange column. After extraction and purification, the fucoidan must be analyzed to evaluate the degree of purity, the composition and also the structure.

1.3.2. Fucoïdan analysis

Various chemical methods are traditionally applied to determine fucoidan structure, although each method has its drawbacks as regards determination of the fine structures (Fig 1.4). For instance, commonly used desulfation methods result in up to 90% fucoidan degradation (Usov and Bilan, 2009). In most publications, fucoidan structure was determined using mass spectrometry. However, this method has a number of limitations due to the tendency for loss of sulfates during the analytical process (Silchenko et al. 2014). The lack of precise information on the structure hinders the study of the relationships between a certain fucoidan structure and its respective bioactivity. An application using enzymes to depolymerize a fucoidan polymer can significantly facilitate the determination of its fine structure (Silchenko et al., 2013), (Silchenko et al. 2017b).

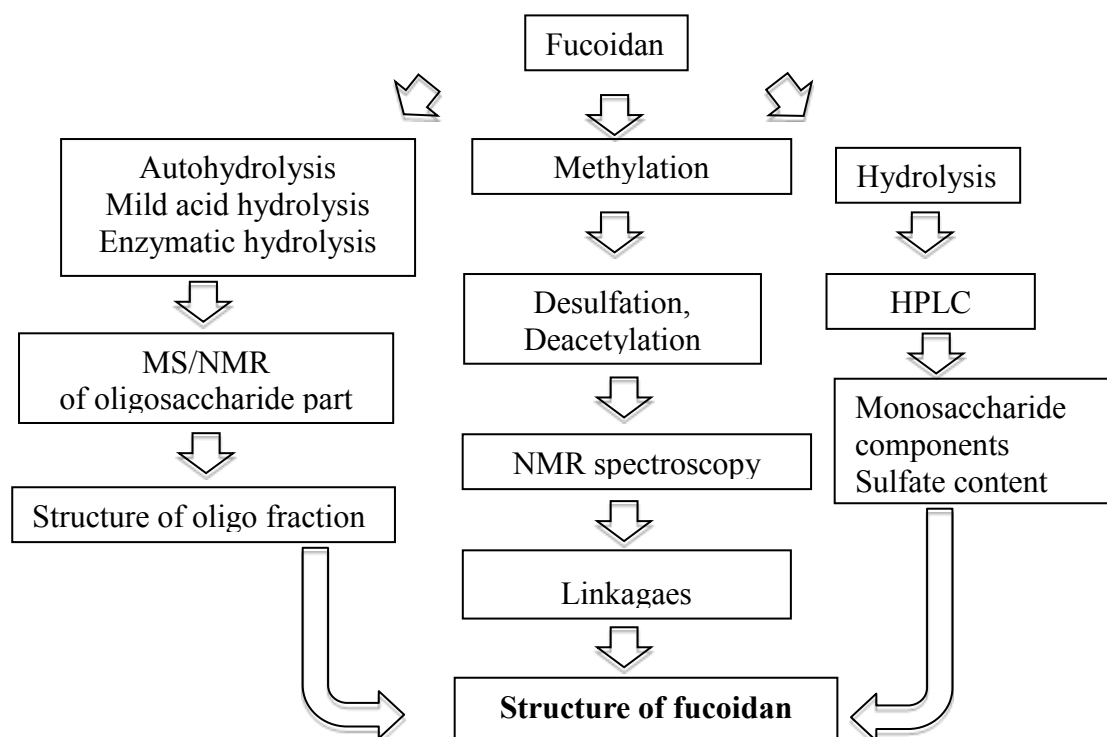


Figure 1.4 Scheme to analyse and identify fucoidan structure

1.4. Fucoidan structure

A large number of publications are devoted to the study of the bioactivities of fucoidan but until now no fucoidans have been registered as a certified medicinal drug. The reason is the heterogeneity of purified fucoidans and the lack of clarity about the structural vs. biological activities. Structural investigation of fucoidans is very difficult because of the large variation in monosaccharide composition, different types of glycosidic linkages and the presence of large numbers of non-carbohydrate substituents (sulfate, methyl, carbonyl groups).

It is now considered that fucoidans are species-specific polysaccharides. This means that each algal species synthesizes fucoidan or a set of fucoidans characteristic only for that species (Ermakova et al. 2015a). Sulfated fucose residues and often galactose are the major constituents of fucoidan. Minor monosaccharide components are mannose, glucuronic acid, xylose, and other less common monosaccharides such as rhamnose (Kusaykin et al., 2008).

To date, several structural groups of fucoidans have been found that differ in the type of O-glycosidic bonds between the residues of α -L-fucose in the polysaccharide:

- Fucoidan constructed solely from residues of α -L-fucose bound together by $\alpha(1\rightarrow3)$ -O-glycosidic bonds. This group occurs in the Laminariales and Ectocarpales orders.

- Fucoidans containing residues of α -L-fucose bound (1 \rightarrow 3)- and (1 \rightarrow 4)-O-glycosidic bonds. They are often synthesized by brown algae of the Fucales order.
- Sulfated galactofucans, the main components of which are residues of α -L-fucose and β -D-galactose bound by (1 \rightarrow 3)-and/or (1 \rightarrow 4)-O-glycosidic bonds. They are usually synthesized by brown algae of the order Laminariales.
- Fucoidans of more complex composition.

The classification of fucoidans is a complex task because one species of brown alga can synthesize α -L-fucans and other fucose-containing polysaccharides such as sulfated galactofucan, fucogalactan, fucoglucuronomannans and xylofucoglucuronans. The different structures of fucoidan might be related to osmotic regulation and species zonation. Fucoidans from algae that grow in temperate regions (Laminariales order) normally have simpler structures than fucoidans from tropical brown algae (Fucales order).

Selected representatives of some algal fucoidans are presented below.

1.4.1. Fucoidan constructed of α (1 \rightarrow 3)-linked sulfated L-fucose

Brown algae of the order Laminariales and Ectocarpales synthesize fucoidans with α (1 \rightarrow 3)-linked sulfated fucose residues as the main structural motive (Fig 1.5). Fucoidans isolated from the Laminariales order are constructed of α (1 \rightarrow 3)-linked fucose residues sulfated at C2 and/or C4 positions. Fucoidans with this structure were isolated from the brown alga *Saccharina cichorioides* (previously *Laminaria cichorioides*) (Zvyagintseva et al., 2003) (Anastyuk et al., 2010a), *Saccharina latissima* (previously *Laminaria saccharina*) (Biland et al., 2010) and *Lessonia vadosa* (Chandía & Matsuhira, 2008)

Fucoidan of fairly simple composition containing practically only fucose, sulfate and acetyl groups has been isolated from the brown alga *Chorda filum*, Laminariales order. The *Chorda filum* fucoidan consists of hexasaccharide repeating units in which five α (1 \rightarrow 3) linked residues of α -L-fucose make up the backbone and one fucose residue is positioned as a side branch at C2. The hydroxyl groups at position C4, and less frequently at C2, are esterified with sulfate groups (Chizhov et al., 1999).

In addition to fucose residues, galactose may also be part of the fucoidan molecule, such as in fucoidans from *Laminaria gurjanovae* (Shevchenko et al., 2007), *Laminaria japonica* (Wang et al., 2010) and *Saccharina longicruris* (Rioux et al. 2010).

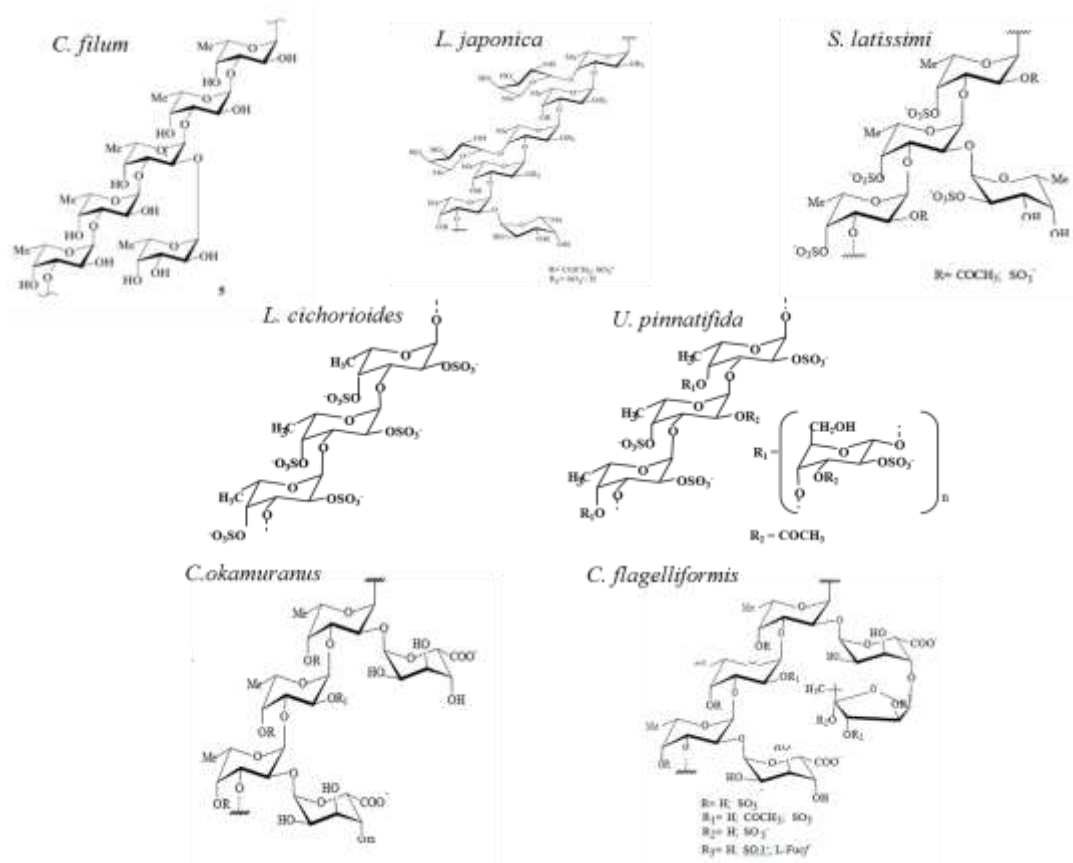


Figure 1.5 Fucoidans from different species of brown algae composed of $\alpha(1 \rightarrow 3)$ -L-fucose: *Chorda filum* (Chizhov et al., 1999), *Laminaria japonica* (Wang et al., 2010), *S. latissimi* (Usov et al., 1998), *L. cichorioides* (Anastyuk et al., 2010) and *U. pinnatifida* (Skriptsova et al., 2010), *Cladosiphon okamuranus* (Sakai et al. 2003), *Chordaria flagelliformis* (Bilan et al., 2008)

There is currently conflicting data concerning the structure of the fucoidan from *Laminaria cichorioides*. According to Zvyagintsev's group, fucoidan from *L. cichorioides* collected in Troitsa Bay, Sea of Japan, consist of linear $\alpha(1 \rightarrow 3)$ -linked residues of α -L-fucose in which α -L-Fucp residues are 2,4-disulfated (Zvyagintseva et al., 2003). This observation is in accord with the suggestion that the Laminariales order (Phaeosporophyceae) has a fucoidan core structure of $(1 \rightarrow 3)$ -linked α -L-fucans (Cumashi et al., 2007). However, another group of researchers has shown that fucoidan from *L. cichorioides* collected in the East Sea (Korea) is a galactofucan (Fuc:Gal = 2:1) in which the α -L-Fucp residues are 2,3-disulfated and $(1 \rightarrow 4)$ -linked (Yoon et al., 2007). Furthermore, in 2010, the structure of fucoidan from *Laminaria cichorioides* collected from Roitsa Bay (Japan Sea, Russia) was shown using tandem MALDI and ESI mass spectrometry to be predominantly linked with the $\alpha(1 \rightarrow 3)$ -type of linkage and to be sulfated mostly at C-2 or C-2/C-4 of the α -L-fucose residues (Anastyuk et al., 2010). Thus, the different

structures of fucoidan from *L. cichorioides* might be dependent on where the algal samples originate.

Four fucoidan fractions have been isolated from the brown alga *S. latissimi*. The first fraction had a structure typical of the Laminariales order: the main chain was constructed of $\alpha(1\rightarrow3)$ -linked fucose residues, sulfated mainly at C4 and less often at C2 positions (Usov et al., 1998). Fucogalactan, fucoglucuronomannan, and fucoglucuronan were also isolated using anion exchange separation (Bilan et al., 2010). A similar fraction of fucoglucuronomannan was isolated from *Kjellmaniella crassifolia* (Sakai et al. 2003).

A fraction of galactofucan was isolated from the brown alga *Undaria pinnatifida*, a species of the Laminariales order, where the main chain was constructed of $(1\rightarrow3)$ -linked residues of α -L-fucose and β -D-galactose in a ratio of 1:0.9. Minor quantities of xylose and mannose were also found in the composition of this polysaccharide (Synytsya et al., 2010). From the same species of algae, sulfated galactofucan was isolated, with the same ratio of α -L-fucose residue to β -D-galactose residues (1:0.9). This polysaccharide was found to consist of blocks containing fucose and galactose residues; residues of α -L-fucopyranose were sulfated at C2 and less frequently at C4, and residues of β -D-galactopyranose were sulfated at C3 and / or C6 (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010).

In contrast to the order Laminariales, the structure of fucoidan from the order Ectocarpales comprises fucoidans with a large number of lateral branches. Thus, fucoidan obtained from *Cladosiphon okamuranus* (Fig 1.5) was shown to have a backbone was constructed from residues of L-fucose connected by $\alpha(1\rightarrow3)$ -O-glycosidic bonds and having lateral branches, mainly in the form of α -D-glucuronic acid at position C2 (Sakai et al. 2003). Sulfate groups were located mainly at C4 in the fucopyranose residue. A more complex structure was found in fucoidan from *Chordaria flagelliformis* in which the backbone was partially glycosylated at position C2 with -D-glucuronic acid (Fig 1.5) (Bilan et al., 2008).

The brown alga *Analipus japonicus*, which is a member of the Ralfsiales order, produces fucoidan of $(1\rightarrow3)$ -linked residues of α -L-fucose. This fucoidan was also found to have branches of predominantly $\alpha(1\rightarrow4)$ - and less often $\alpha(1\rightarrow2)$ -bound residues of L-fucopyranose (Biland et al., 2007). The sulfate groups were located mainly in the C2 position, and the acetyl groups at the C4 position of the fucopyranose residue in the backbone.

1.4.2. Fucoidan constructed of α -L-fucose linked (1 \rightarrow 3) and (1 \rightarrow 4)

Fucoidan with (1 \rightarrow 4) glycosidic linkages between L-fucose residues is less common and is present mainly as α (1 \rightarrow 3); α (1 \rightarrow 4)-L-fucans (Chevolot et al., 2001), (Bilan et al., 2002) (Descamps et al., 2006). Fucoidans of this structure are mainly synthesized by the family Fucaceae of the order Fucales, including *Fucus vesiculosus*, *Fucus evanescens*, *Fucus distichus*, *Fucus serratus*, *Ascophyllum nodosum* (Chevolot et al., 2001) and *Pelvetia canaliculata* (Descamps et al., 2006).

The first fucoidan of this type was isolated from the brown alga *F. vesiculosus* (Conchie & Percival, 1950). However, because of the large number of substituents in the molecule, its structure was not established correctly. Conchie and Percival claimed that the fucose residues in the fucoidan molecule were connected by α (1 \rightarrow 2)-O-glycosidic bonds and sulfated at the C4 position (Conchie & Percival, 1950). This model of the fucoidan structure persisted for about 40 years. In 1993, the structure of fucoidan from *F. vesiculosus* was revised (Patankar et al., 1993). The newly proposed structure suggested that the fucose residues were linked by α (1 \rightarrow 3)-O-glycosidic bonds, and in addition contained branching at the C2 position and sulfate groups at C4. Subsequently, refinements were made to the structure of this fucoidan. It was furthermore shown that the backbone contains a repeating motif consisting of sulfated fucose residues bound by alternating α (1 \rightarrow 3)- and α (1 \rightarrow 4)-glycosidic bonds, and sulfate groups are at C2 and to a lesser extent at C3 of the L-fucose residues (Chevolot et al., 2001). An analogous structure was found in the fucoidans from *A. nodosum* (Chevolot et al., 2001) and *P. canaliculata* (Descamps et al., 2006).

Fucoidans obtained from *F. evanescens* of the Fucaceae are sulfated fucans with a main chain of alternating residues of (1 \rightarrow 3)-and (1 \rightarrow 4)-linked α -L-fucose (Chevolot et al., 2001) (Bilan et al., 2002). The structure of fucoidan from *F. evanescens* was studied by two research groups: Zvyagintseva et al. (Zvyagintseva et al., 2003), (Kusaykin et al., 2006), (Anastyuk et al., 2009), (Silchenko et al., 2014), (Kusaykin et al., 2003) and Bilan et al. (Maria I Bilan et al., 2002). Bilan et al. found that this fucoidan had a linear structure with alternating glycoside bonds of (1 \rightarrow 3)-and (1 \rightarrow 4) characteristic for this group of fucoidans, with sulfate groups attached at C2 and sometimes at C4, and randomly distributed acetyl groups. In 2003, Kusaykin et al compared fucoidans that they had isolated and fucoidans from the Bilan group. Their fucoidan contained more α (1 \rightarrow 3)-linked fucose residues (Kusaykin et al., 2003). The differences may

have come from the purification procedures or could have been due to harvest of the algal samples at different places or at different seasons.

Structural characteristics of fucoidan from *Stoechospermum marginatum*, an alga of the order Dictyotales, have also been established. Fucoidan of this species was found to be constructed of fucose residues linked by $\alpha(1\rightarrow3)$ - and $\alpha(1\rightarrow4)$ -O-glycosidic bonds and sulfated at the C2 and/or C4 positions (Adhikari et al., 2006).

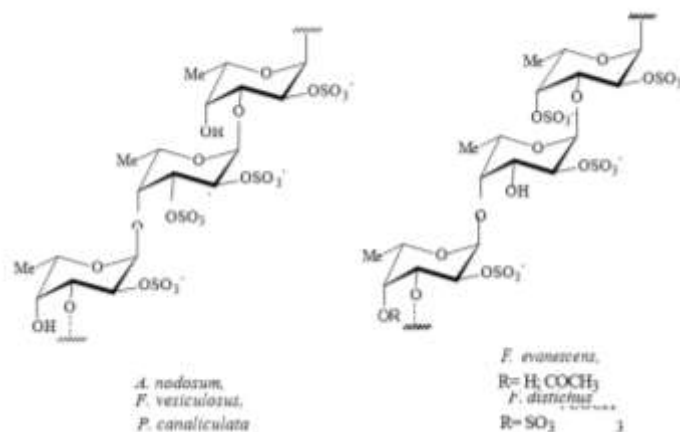


Figure 1.6 Fucoidans constructed from $\alpha(1\rightarrow3)$ - and $\alpha(1\rightarrow4)$ -L fucose residues

1.4.3. Sulfated galactofucans

Many fucoidans contain small amounts of other monosaccharides apart from fucose, but the linkages and positions of these minor components are often unknown. However, polysaccharides that consist of approximately equal amounts of sulfated fucose and galactose residues are commonly called galactofucans and the galactose residues are most often dispersed throughout the backbone. The position and amount of galactose residues in various galactofucans depend on the type of algae (Bilan et al., 2013), (Pham et al., 2013). This is the most structurally diverse group of fucoidans.

The structures of the sulfated galactofucans from *Sargassum polycystum*, *Sargassum mcclurei*, *Sargassum duplicatum* and *Turbinaria ornata* from the Vietnam Sea have also been investigated. They are all galactofucans with complex structures. The *S. mcclurei* fucoidan is essentially a sulfated galactofucan polysaccharide with both $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ linked fucosyl residues and galactosyl- $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages in the backbone. The fucosyl residues in *S. mcclurei* fucoidan are differentially sulfated at C2 and/or at C4, and some of the galactosyl moieties are even sulfated at C6 and have a sulfate content of up to 35% (Pham et al., 2013). Fucoidan extracted from *T. ornata* collected at Nha-Trang bay, Vietnam, can also be categorized as a

galactofucan. The backbone of *T. ornata* fucoidan has thus been proposed to consist of $\alpha(1\rightarrow3)$ -linked L-fucosyls with galactosyl branches (Fuc:Gal \approx 3:1) and a high sulfate content of \sim 25% with the sulfate attached mostly at C2, and to lower degree at C4, of both the fucosyl and the galactosyl residues (Thanh et al., 2013) (Ermakova et al. 2015b).

1.4.4. Fucoidans of more complex composition

A small group of fucoidans is represented by fucomannuronans (Imbs et al., 2011). Furthermore, there are fucoidans of more heterogeneous monosaccharide composition.

Thus fucoidan have a diversity of structures which depend on the sources brown seaweed as well as the place where the seaweeds were harvested. But purified fucoidan of high molecular weight and high viscosity has not yet been applied in the medicinal industry, as mentioned above. One possible solution would be to create sulfated fuco-oligosaccharides with identifiedbiologically activites. In this way the relationship between the bioactivity and the precise fucoidan structures could be established. And enzymes with known specificity that catalyze fucoidan hydrolysis (fucoidan hydrolases, sulfatases) are the best tool with which to obtain fuco-oligosaccharides without changing the native structure of the fucoidans.

Table 1.2 Structure characteristics of some fucoidans

Sources	Type of fucoidan	Composition	Main structure	Position of SO ₄ group
<i>Laminaria cichorioides</i> (Anastyuk et al., 2010)	(1→3)	Fuc:man = 1:0.05	[3)- α -L-Fucp-(2,4OSO ₃)-1→3- α -L-Fucp-(2,4OSO ₃)-(1→]	C2/ C4
<i>Fucus evanescens</i> from Pacific sea (Bilan et al., 2002)	(1→3)- and (1→4)		[→3)- α - L -Fucp-(2SO ₃ ⁻)-(1→4)- α -L-Fucp-(2SO ₃ ⁻)-(1→]n partly sulfated at C4, acetylated; 1→3:1→4 = 1:1	Mainly at C2
<i>Fucus evanescens</i> (Kusaykin et al. 2006)	1→3)- and (1→4)	Fuc :Xyl : Man: Glu = 1: 0.03: 0.002:0.02	[→3)- α -L-Fucp-(2SO ₃ ⁻)-(1→4)- α -L-Fucp-(2SO ₃ ⁻)-(1→]n partly sulfated at C4; 1→3:1→4 = 3:1	Mainly at C2
<i>Fucus vesiculosus</i> (Chevolot et al., 2001)	(1→3)- and (1→4)-		[→3)-L- α -Fucp-(2SO ₃ ⁻)-(1→4)- α -L-Fucp-(2SO ₃ ⁻)-(1→]n	C2 and C2/C3
<i>Sargassum mcclurei</i> Viet Nam (Pham et al., 2013)	Galacto- fucan	Fuc:Gal = 1:0.6	Main chains: →3)- α -L-Fucp-(2,4-SO ₃ ⁻)-(1→ including residues of →4)- α -L-Fucp-(3-SO ₃ ⁻)-(1→ and →6)-Gal Branches consist of alternating fucose and galactose residues	C2/C4 or C2 ,C4
<i>Turbinaria ornata</i> (Vietnam) (Ermakova et al. 2015b)	Galacto- fucan	Fuc:Gal = 1:0.2	The main chain is →3)- α -L-Fucp-(1→ Single residues or short chains of fucose and galactose in the branches	C2 / C4
<i>Undaria pinnatifida</i> (Lee et al., 2004)	Galacto- fucan	Fuc:Gal =1.0: 1.1.	The main chain is α (1→3)- linked galactosyl residues	C2 / C4

1.5. Fucoidan modifying enzymes

A small group of fucoidans is represented by fucomannuronans (Imbs et al., 2011). Furthermore, there are fucoidans of more heterogeneous monosaccharide composition.

Thus fucoidan have a diversity of structures which depend on the sources brown seaweed as well as the place where the seaweeds were harvested. But purified fucoidan of high molecular weight

and high viscosity has not yet been applied in the medicinal industry, as mentioned above. One possible solution would be to create sulfated fuco-oligosaccharides with identified biological activities. In this way the relationship between the bioactivity and the precise fucoidan structures could be established. And enzymes with known specificity that catalyze fucoidan hydrolysis (fucoidan hydrolases, sulfatases) are the best tool with which to obtain fuco-oligosaccharides without changing the native structure of the fucoidans.

1.5.1 Sources of fucoidanases and fucoidan sulfatase

In marine ecosystems, fucoidans from brown seaweeds are a rich carbon source for different organisms, and it is likely that such organisms, e.g. invertebrates, bacteria and fungi, would be able to degrade the fucoidans. Sea urchins (Echinoidea) and abalones (Gastropoda) mainly ingest algae, with a preference for brown algae. Fucoidan-degrading enzymes are produced by algal feeders such as *Patinopecten yessoensis* (Kitamura et al., 1992), marine mollusks *Haliotus* sp. (Thanassi & Nakada, 1967), *L. sitkana* (Bilan et al., 2005), sea urchin *S. nudus* (Sasaki et al. 1996), (Furukawa et al. 1992), and Vietnamese mollusk *Lambis* sp. (Silchenko et al., 2014). Fucoidanases are also produced by bacteria associated with brown seaweeds, sea cucumbers and sea urchins (Furukawa et al. 1992) (Bakunina et al., 2000) (Sakai et al. 2004). To date, fucoidanases have been isolated from the marine bacteria *Fucophilus fucoidanolyticus* SI-1234 (Sakai et al., 2003a), *Flavobacterium* sp. F-31, *Flavobacteriaceae* CZ1127, *Sphingomonas paucimobilis* PF-1, *Alteramonas* sp. SN-1009, *Fucobacteriaceae* SA-0082, *Vibrio* sp. N-5, *Pseudoalteromonas citrea* KMM 3296, KMM 3297, KMM 3298, and from marine fungi *Dendryphiella arenaria* TM94 and *Fusarium* sp. LD8 (Table 1.3).

Table 1.3 Fucoidan backbone modifying enzymes from marine organisms

Sources of enzymes	Substrate	Enzymes	Products	Reference
Invertebrates				
<i>Haliotis sp.</i>	<i>Fucus gardneri</i>	Nd	Fucose and Fucooligosaccharides	(Thanassi & Nakada, 1967)
<i>Strongylocentrotus nudus</i>	2-sulfo- α -L-fucopyranosyl-(1 \rightarrow 2) pyridylaminated fucose	exo, α (1 \rightarrow 2)	2-sulpho- α -L-fucopyranose	(Sasaki et al. 1996)
<i>Mizuhopecten yessoensis</i>	<i>Nemacystus decipiens</i>	Nd	Fucooligosaccharides	(Kitamura et al. 1992)
<i>Pecten maximus</i>	<i>Ascophyllum nodosum</i>	Fucosidase (exo-fucoidanase)	Fucose and sulfated Fucooligosaccharides contained α (1 \rightarrow 3), α (1 \rightarrow 4)- linked fucose residues	(Berteau et al., 2002)
<i>Littorina sitkana</i>	<i>Fucus distichus</i> (Fucose:SO ₄ :Acetyl = 1:1.21:0.08)	Endo 1-3	Sulfated fucooligosaccharides contained α (1 \rightarrow 3), α (1 \rightarrow 4)- linked fucose residues	(Bilan et al., 2005)
<i>Lambis sp.</i>	<i>F. evanescens</i>	endo, α -1 \rightarrow 4	Sulfated fucooligosaccharides contained α (1 \rightarrow 3), α (1 \rightarrow 4)-linked fucose residues	(Silchenko et al., 2014)
Bacteria				
<i>Vibrio sp.</i> N-5	<i>Kjellmaniella crassaifolia</i>	Exo-type	Sulfated fucose and/or sulfated fucobiose	(Furukawa et al. 1992)
" <i>Fucobacter marina</i> " SA-0082	<i>K. crassifolia</i>	endo, β -1 \rightarrow 4 between GlcUA Manp residues	unsaturated sulfated fucoglucuronomannooligosaccharides	(Sakai et al. 2002, 2003b)
<i>Alteromonas sp.</i> SN-1009	<i>K. crassifolia</i>	endo, α -1 \rightarrow 3	Sulfated fucooligosaccharides contained α -1 \rightarrow 3 linked fucose residues sulfated	(Sakai et al., 2004)
<i>Pseudoalteromonas citrea</i> KMM 3296, KMM 3297, KMM 3298	<i>Fucus evanescens</i> <i>Laminaria cichorioides</i>	endo, α -1 \rightarrow 3	Sulfated fucooligosaccharides	(Bakunina et al., 2002)

<i>"Fucophilus fucoidanolyticus"</i> SI-1234	<i>Cladosiphon okamuranus</i>	endo, α -1 \rightarrow 3	fucooligosaccharides containing α -1 \rightarrow 3 linked fucose with GlcUA branches linked 1 \rightarrow 2 to fucose	(Sakai et al., 2003a)
<i>Mariniflexile fucanivorans</i> SW5	<i>Pelvetia canaliculata</i>	Endo α - 1 \rightarrow 4	Tetrasaccharide Hexasaccharide	(Descamps et al., 2006)
<i>Flavobacteriaceae</i> CZ1127	<i>Acaudina molpadioides</i> From sea cucumber	endo, α -1 \rightarrow 3	Sulfated fucooligosaccharides made of α -1 \rightarrow 3 linked sulfated fucose residues	(Chang et al., 2010) (Yu et al., 2013)
<i>Formosa algae</i> KMM 3553T	<i>F. evanescens</i>	endo, α -1 \rightarrow 4	Sulfated Fucooligosaccharides	(Silchenko et al., 2013)
<i>Sphingomonas paucimobilis</i> PF-1	<i>Undaria pinnatifida</i>			(Kim et al., 2015)
Marine fungi				
<i>Dendryphiella arenaria</i> TM94	<i>Fucus vesiculosus</i> and <i>Laminaria digitata</i>	Endo, nd	Oligosaccharides	(Wu et al., 2011b)
<i>Fusarium</i> sp. LD8	<i>F. vesiculosus</i>	Endo, nd	Oligosaccharides	(Wu et al., 2011a)

Fucoidan sulfatases also have been found in marine bacteria such as *Vibrio* sp. N-5 (Furukawa et al. 1992) and marine invertebrates such as in the digestive glands of a sea mollusk (Berteau et al., 2002). Other bivalve and gastropod mollusks and also many representatives of other types of marine invertebrates often contain highly active arylsulfatases; examples are *Littorina kurila* (Kusaykin et al., 2003) and *Turbo* sp. (Pesentseva et al., 2012) which were reported to produce fucoidan sulfatases.

Thus the marine environment is rich in fucoidanases and fucoidan sulfatases which are frequently found in marine organisms. Among them, sea cucumbers serve a useful role in the marine ecosystem as they help recycle nutrients by breaking down detritus and other organic matter which bacteria can then continue to degrade further (Du et al., 2012). So sea cucumber could be an interesting subject in which to find fucoidanase and sulfatase. Proteobacteria and Bacteroidetes spp. associated with sea cucumber were reported to be producers of fucoidanases (Bakunina et al., 2000). On the other hand, the envelope of sea cucumbers contains fucoidans (Chang et al., 2010) and therefore the associated bacteria may possess enzymes capable of degrading these polysaccharides. For this reason, screening for bacteria producing fucoidan

modifying enzymes (fucoidanases and sulfatases) in sea cucumber gut was employed as a part of this thesis.

Sea cucumbers are marine invertebrates and they are belonging to the class *Holothuroidea*. There are about 1,717 holothurian species (Anderson et al., 2011) with the greatest number being in the Asia Pacific region (Du et al., 2012). Sea cucumbers found in Vietnam (at least in Khanh Hoa Province) appear to be closer to *Holothuri scabra* in terms of their small size and size at first maturity (Fig 1.7). The color of sea cucumbers ranges from black through dark brown to light beige, often with transverse stripes. They also appear less deeply ridged than many pictured from Oceania. The species may have potential for commercial aquaculture and for restocking or stock enhancement. In Nha Trang bay *Holothuri scabra* are co-existing with brown seaweeds *S.mcclurei* and *T.ornata*, therefore they also have potential to find fucoidan – modifying enzymes.

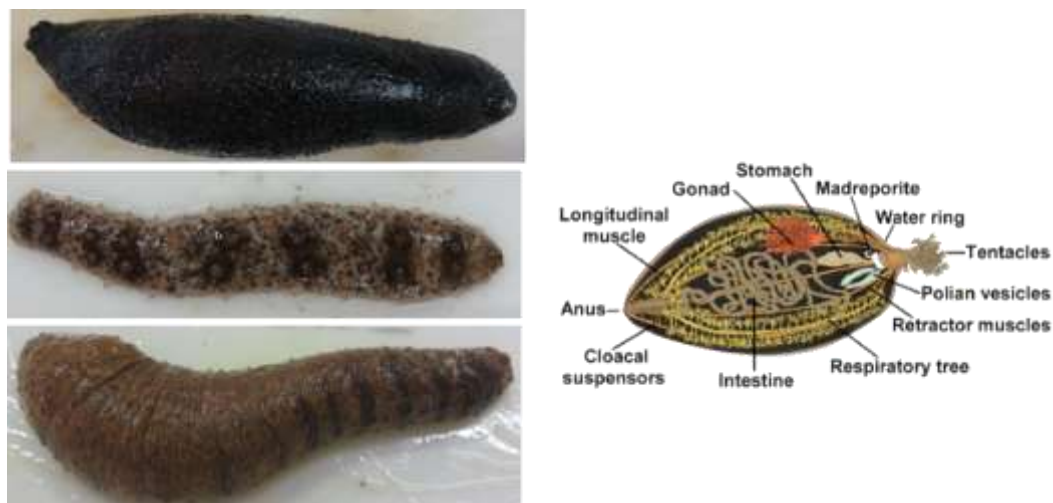


Figure 1.7 Phenotypic variation in *Holothuria scabra* in Nhatrang Bay, Vietnam.

1.5.2. Fucoidanases

1.5.2.1. Nomenclature and classification of fucoidanases

Enzymes that catalyze fucoidan are called fucoidan hydrolases. According to Berteau and Mulloy (Berteau & Mulloy, 2003) there are at least two types of glycosidases that catalyze degradation of fucoidan: fucan sulfate hydrolases also called fucoidanases, and fucosidase. The activity of α -L-fucosidases is easily described as the release of L-fucose from the non-reducing end of a polysaccharide. The activity of fucoidanase is described as endo-acting on the fucoidan back bond and the products are oligosaccharides.

It is a very difficult task to correctly classify fucoidanases because of the complex structure of native fucoidans which are often heteropolysaccharides as we discussed above. Data on the

fucoïdan structure is unclear and sometimes incorrect lead to confusion when classifying the enzymes. For example, the structure of fucoïdan isolated from *Fucus vesiculosus* was incorrectly described as $\alpha(1\rightarrow2)$ -L-fucan (O'Neill, 1954), which led to misclassification of fucoïdanases belonging to EC 3.2.1.44 that were described as $(1\rightarrow2)$ fucoïdanases. Later, the description of *F. vesiculosus* fucoïdan structure was altered to the correct alternating $\alpha(1\rightarrow3)$ - and $\alpha(1\rightarrow4)$ -linked fucose structure (Patankar et al., 1993). However, the enzyme classification of EC 3.2.1.44 has not yet been changed.

Enzyme classification based on amino acid sequence similarities is an alternative to that based on specific activities. The classification based on homology is used in the Carbohydrate Active Enzyme or CAZy database (Lombard et al., 2014). According to this classification, fucoïdanases belong to family GH107 of glycoside hydrolases. The fucoïdanase FcnA (CAI47003.1) isolated from the marine bacterium *Mariniflexile fucanivorans* SW5T (Colin et al., 2006) is the first characterized member of this family. In 2017, two endo-fucoïdanases, FFA1 and FFA2, from the marine bacterium *Formosa algae* (KMM 3553T) were reported to belong to GH107. FcnA, FFA1 and FFA2 are endo $(1\rightarrow4)$ fucoïdanases and they were isolated from bacteria belonging to the phylum Bacteroidetes. Other members of GH107 are five sequences from *Wenyngzhuangia fucanilytica* that also belong to Bacteroidetes. Two fucoïdanases isolated from [*Alteromonas*] sp., SN-1009 Fda1 (AAO00508.1) and Fda2 (AAO00509.1) (Takayama 2002), also belong to family GH107. [*Alteromonas*] sp. SN-1009 belongs to the class Gammaproteobacteria which are very distant to Bacteroidetes and this enzyme was reported to be endo $(1\rightarrow3)$ fucoïdanase. SVI_0379 from *Shewanella violacea* DSS12^T, Gammaproteobacteria, might be endo $(1\rightarrow3)$ fucoïdanase. Thus, members of the phylum Bacteroidetes have potential for producing endo $(1\rightarrow4)$ fucoïdanase and members of Gammaproteobacteria have potential for producing endo $(1\rightarrow3)$ fucoïdanase.

Apart from the 10 members of family GH107, Sakai et al. as early as in 2003 reported the discovery of a new type of extracellular endo-fucoïdan-lyase activity from “*Fucobacter marina*” SA-0082, or more correctly *Flavobacterium* sp. SA-0082, which acted on sulfated fucoglucurono-mannan from *K. crassifolia* (*S. sculpera*) (Sakai et al. 2003b). Using sequence analysis this lyase activity was found to be apparently encoded by two separate coding regions. Recombinant expression of these two putative fucoïdan degrading enzymes, referred to as FdIA and FdIB, respectively, showed that the two enzymes had about 56% amino acid sequence identity and both were claimed to act as (glucurono-) fucoïdan lyases on *K. crassifolia* (*S. sculpera*) fucoïdan (Takayama et al., 2002).

Table 1.4 Fucoidan degrading enzymes in GenBank

Protein Name	Amino acid	Organisms	GenBank
Fucanase (Fda1)	814	<i>Alteromonas</i> sp. SN-1009	AAO00508.1
Fucanase (Fda2)	881	<i>Alteromonas</i> sp. SN-1009	AAO00509.1
Sulfated fucan endo- α -1,4-L-fucanase (FcnA)	1007	<i>Mariniflexile fucanivorans</i> SW5	CAI47003.1
SVI_0379	574	<i>Shewanella violacea</i> DSS12	BAJ00350.1
AXE80_07420	967	<i>Wenyingzhuangia fucanilytica</i> CZ1127	ANW96115.1
AXE80_07425	799		ANW96116.1
AXE80_07310	883		ANW96098.1
AXE80_07305	800		ANW96097.1
poly[(1 \rightarrow 4)- α -L-fucoside-2-sulfate] glycano hydrolase FFA2	917	<i>Fomosa algae</i> KMM 3553T	WP_057784219.1
FFA1	1008	<i>Fomosa algae</i> KMM 3553T	WP_057784217.1
FdlA	704	<i>Flavobacterium</i> sp. SA-0082	AAO00510.1
FdlB	697	<i>Flavobacterium</i> sp. SA-0082	AAO00511.1

1.5.2.2. Structure and predicted domains of bacterial fucoidanases

To date, only fucoidanase sequences from bacteria are available. The fucoidanases originate from species of bacteria of the phylum Bacterioidetes, except Fda1, Fda2 and SVI, which were isolated from the Gammaproteobacteria. The sequences of the fucoidanases cluster together mostly in relation to the category of bacteria they originate from. A similar sequence has not yet been identified in other categories of bacteria, even though several have been shown to have fucoidanase activity. The fucoidanases presented in Table 1.4 are enzymes 574-1008 amino acids in size. They contain several predicted domains, primarily in the C-terminal, but also contain N-terminal excretion signals.

According to BLASTp and PSI-BLAST, the amino acid sequences of fucoidanases from the different bacterial groups did not have a high degree of similarity (Table 1.5).

Table 1.5 Amino acid sequences identity degree of the currently known fucoidanases

Fucoidanase (sources)	Identity, %									
	FcnA (1)	FFA1 (2)	FFA2 (3)	AXE80				Fda1 (8)	Fda2 (9)	SVI_03 937 (10)
				07305 (4)	07425 (5)	7420 (6)	7310 (7)			
(1)	100	56	67	49	70	39	39	20	21	21
(2)	56	100	57	49	72	39	39	18	19	21
(3)	67	57	100	48	55	40	43	18	20.	20
(4)	49	49	48	100	45	39	49	16	15	15
(5)	70	72	55	45	100	65	42	16	15	25
(6)	39	39	40	39	65	100	45	14	14	12
(7)	39	39	43	49	42	45	100	10	11	14
(8)	20	18	18	16	16	14	10	100	71	32
(9)	21	19	20	15	15	14	11	71	100	31
(10)	21	21	20	15	25	12	14	32	31	100

FcnA and FFA1, FFA2 showed the highest percentage of amino acid sequence identity with 56.26% and 66.77%, respectively. However, FcnA showed the low percentage of identity with Fda1 (19.76%), Fda2 (21%) and SVI_03937 (21.40%) (Table 1.5). This point can be explained by their different group of bacteria and substrate specificity: FcnA cleaves $\alpha(1\rightarrow4)$ -glycosidic bonds, and Fda1 and Fda2 cleave $\alpha(1\rightarrow3)$ -glycosidic bonds.

Analysis of the hydrophobic clusters (AGCs) of amino acid sequence sections (Lemesle-Varloot et al., 1990) of FcnA and Fda2 revealed a high degree of similarity of their N-terminal fragments, which indicates common elements in the secondary structure of these polypeptide sites though the amino acid sequences show low identity (Fig 1.8) (Colin et al., 2006).

Analysis of the amino acid sequence of FcnA via the RADAR server (Heger and Holm, 2000) revealed three repeating domains, each with a length of about 105 amino acid residues. According to the Pfam database (Finn et al., 2014), these domains belong to the family of cadherin-like domains (Fig 1.9). Cadherins are representatives of a large family of calcium-dependent proteins that participate in cell adhesion in higher organisms and affect tissue morphogenesis (Tepass et al., 2000). The function of cadherins and cadherin-like domains in

proteins synthesized by bacteria remains poorly understood. Presumably, cadherin domains participate in cell adhesion through protein-protein interactions (Cao et al., 2005) and /or bind to polysaccharides (Fraiberg et al., 2010), (Fraiberg et al., 2012). There are publications about the presence of cadherins and cadherin-like domains in lectins of the bacterium *Saccharophagus degradans* 2-40, which perform calcium-dependent binding with various polysaccharides (Fraiberg et al., 2012). Fraiberg concluded that a repeated doublet of cadherins or cadherin-like domains in the enzymes of *S. degradans* 2-40 performs the function of binding to polysaccharides.

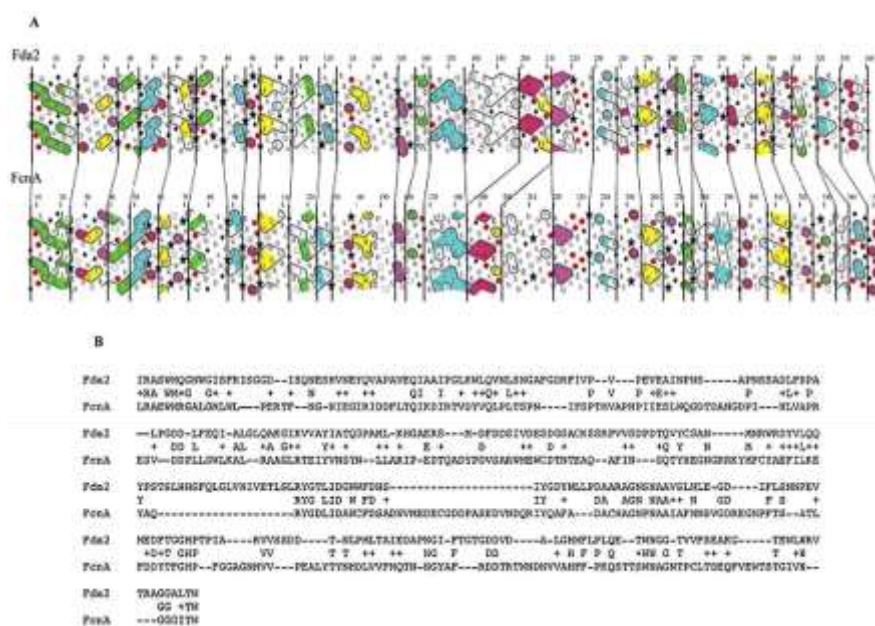


Figure 1.8 N-terminal catalytic domain of FcnA fucanase from *M. fucanivorans* SW5. (A) hydrophobic cluster analysis (ACG) of the N-terminal catalytic domain of FcnA from *M. fucanivorans* SW5 and Fda2 from *Alteromonas* sp. SN-1009; (B) Alignment of the amino acid sequences of the N-terminal domain of FcnA and Fda2 fucanases (Colin et al., 2006).

The C-terminal FcnA amino acid sequence of 75 amino acid residues has a high percentage of identity with the C-terminal regions of some proteins, mainly carbohydrases and proteases from bacteria belonging to the Bacteroidetes phylum. This domain does not contain the catalytic function.

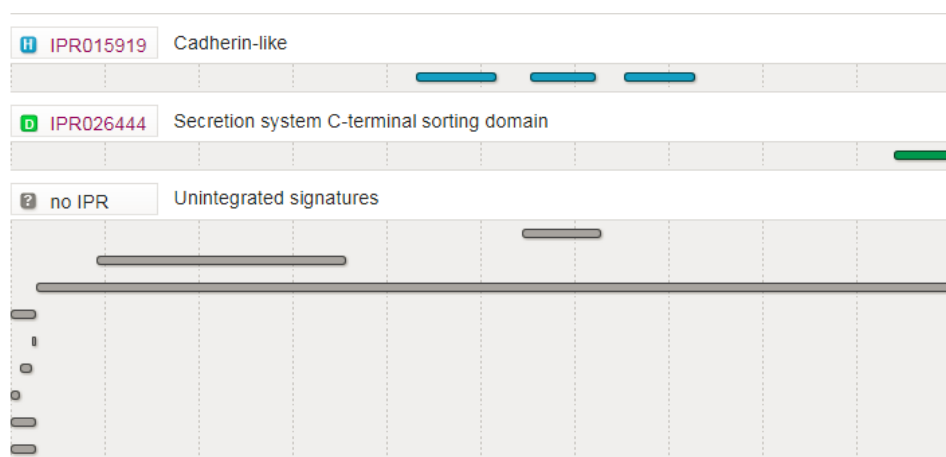


Figure 1.9 Three domains of FcnA2 that belong to the family of cadherin-like domains

The secondary structure of the fucoidanase isolated from the marine fungus *Fusarium* sp. LD8 has been studied using IR-spectroscopy (Wu et al. 2011a). The secondary structure of the enzyme was shown to have three regions containing β -sheet (21.1%, 19.8%, 17.7%), one region corresponding to the α -helix (11.5%), two regions corresponding to β -rotation (9.06%, 6.33%), and a disordered region. Therefore, the fucoidanase structures have not known completely yet.

1.5.2.3. The biochemical properties of fucoidanases

The properties of most fucoidanases have been studied on partially purified enzyme preparations and often using partially purified substrates. Fucoidanases isolated from marine invertebrates had pH optima in the acidic region (Table 1.5). For example, fucoidanases isolated from the mollusks *Haliotis* sp. (Thanassi & Nakada, 1967) and *Mizuhopecten yessoensis* (Kitamura et al. 1992) and sea urchin *Strongylocentrotus nudus* (Sasaki et al., 1996) showed maximum activity in the pH range 3.0 to 5.5. An exception is the fucoidanase from liver of *Littorina sitkana* that had two pH-optima, namely 5.4 and 8.5 (Bilan et al., 2005). The known optimal temperatures of fucoidanases of marine invertebrates lie in the region of 38-45°C, and molecular masses are spread over a wide range from 85 to 200 kDa.

There are few publications on fucoidanases from marine fungi. To date, fucoidanases from *D. arenaria* TM94 (Wu et al. 2011b) and *Fusarium* LD8 (Wu et al., 2011a) have been isolated and characterized. The maximum activity of fucoidanases from marine fungi was shown to occur at pH 6.0 and at 50-60°C. The molecular weight of the fucoidanase from *D. arenaria* TM94 was 180 kDa, while the fucoidanase from *Fusarium* LD8 had a molecular weight of 64 kDa. The fucoidanase from *D. arenaria* TM94 was stable over the pH range 6.0 to 7.0; the enzyme had a half-life of 1 hour at 56°C. Fucoidanase from the marine fungus *Fusarium* LD8 was more

sensitive to changes in temperature; the half-life was 1 hour at 50°C and the enzyme remained stable at pH 6.0.

In contrast to the enzymes of mollusks and marine fungi, fucoidanases of bacteria have an optimum of action in the neutral or alkaline pH region. Enzymes produced by the marine bacterium *Vibrio* sp. N-5 remain active up to 50-60°C and have an optimal temperature of 38-45°C (Furukawa et al. 1992), whereas the fucoidanase from *Flavobacterium* sp. SA-0082 has an optimal temperature of 40°C (Sakai et al. 2003b). Optimal conditions of the fucoidanase from *M. fucanivorans* SW5 were 20-25°C and pH 7.5 (Descamps et al. 2006).

The effect of inhibitors and activators on fucoidanase activity has not been studied *in vivo*, although there are several publications devoted to the study of the effect of metal ions on fucoidanase activity. Heavy metal ions such as Cu^{2+} and Zn^{2+} inhibited fucoidanases from the marine bacteria *Flavobacterium* sp. SA-0082 and *Alteramonas* sp. SN-1009. Fucoidanases of the marine bacterium *Vibrio* sp. N-5 (Furukawa et al. 1992) and the marine mollusk *Haliotis* sp. were inhibited by Ag^+ ions and the divalent metal ions Hg^{2+} , Fe^{2+} and Mn^{2+} . The activities of fucoidanases are activated by Co^{2+} , Mg^{2+} , Ca^{2+} and NaCl (Table 1.6)

1.5.2.4. Type of action and specificity of fucoidanases

Fucoidanases are the least studied among enzymes that act on polyanionic polysaccharides. The mechanism of action of fucoidanases has practically not been investigated. The type of action of fucoidanases, like other glycosidases can be divided into exo-fucoidanases, which cleave sulfated or non-sulfated fucose from the non-reducing end of the fucoidan molecules., and endo-fucoidanases, which attack the internal bonds of the substrate to form different size of oligo products .Most known fucoidanases are enzymes of endo-type action (Table 1.6). Traditionally, various substrates with a known structure have been used to determine the substrate specificity of the enzymes. Information on the type of action of these enzymes can be obtained by establishing the structure of the products of enzymatic hydrolysis by fucoidans.

Fucoidanase FcnA isolated from the marine bacterium *M. fucanivorans* SW5 is the most studied (Descamps et al., 2006) (Colin et al., 2006). To establish the specificity of this enzyme, the authors used a fucoidan substrate from the brown algae, *P. canaliculata*, consisting of repeating disaccharide units of sulfated fucose linked by alternating $\alpha(1\rightarrow3)$ - and $\alpha(1\rightarrow4)$ -glycosidic bonds: $[3)\text{-}\alpha\text{-L-Fucp-(2OSO}_3^-)\text{-1}\rightarrow 4\text{-}\alpha\text{-L-Fucp- (2,3OSO}_3^-)\text{-(1}\rightarrow]$. As a result of the enzymatic reaction, tetra- and hexasaccharides of the following structure were obtained: $[3)\text{-}\alpha\text{-L-Fucp-(2OSO}_3^-)\text{-1}\rightarrow 4\text{-}\alpha\text{-L-Fucp-(2,3OSO}_3^-)\text{-(1}\rightarrow]$ n. Additional information on the substrate specificity

of FcnA fucoidanase was obtained using recombinantly expressed enzymes. To establish the specificity of the enzyme, the NMR spectra of the original fucoidan from *P. canaliculata* and the total fraction of low molecular weight products obtained by enzymatic hydrolysis of this fucoidan were compared. Based on the data obtained, the authors concluded that fucoidanase from *M. fucanivorans* SW5 is specific for $\alpha(1\rightarrow4)$ glycosidic bonds located within the repeating fragment $[4) -\alpha\text{-L-Fucp-}(2,3\text{OSO}_3^-)-1\rightarrow 3-\alpha\text{-L-Fucp-}(2\text{OSO}_3^-)-(1\rightarrow]$ chain of fucoidan. The fucoidanase was a hydrolase because, in the enzymatic hydrolysis of fucoidan, there was no increase in absorption in the UV region of the spectrum at 232 nm characteristic of 4,5-unsaturated pyranose products which accumulate during the cleavage of substrates by a lyase mechanism (Colin et al., 2006).

The structure of the low-molecular products obtained by the action of the extracellular enzymes from *Alteromonas* sp. SN-1009 on fucoidan from *Kjellmaniella crassifolia* was determined (Sakai et al. 2004). Comparison of the structure of the products with the structure of the initial substrate led to the suggestion that the fucoidanases studied were endo-acting enzymes specific for $\alpha(1\rightarrow3)$ -glycosidic bonds. However, the biochemical properties of the recombinant enzymes Fda1 and Fda2 from *Alteromonas* sp. SN-1009 have not yet been characterized.

Enzymes specific for $\beta(1\rightarrow4)$ -glycosidic bonds between glucuronic acid and mannose residues were isolated from the marine bacteria *Flavobacterium* sp. SA-0082 (Sakai et al. 2003b). The enzymes were digested with fucoidan SFGM (fucoglucuronomannan sulfate) from *K. crassifolia* and sulfated 4,5-unsaturated fucoglucuronomannan oligosaccharides were obtained. Almost all products had 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the non-reducing end, and mannose or its sulfated derivatives at the reducing end. The structure of the products indicated that these enzymes were lyases and cleaved the substrate by the β -elimination mechanism.

A gene that encodes the fucoidanase FFA2 from the marine bacterium *Formosa algae* strain KMM 3553T was cloned, and the protein (FFA2) was produced in *Escherichia coli* (Silchenko et al. 2017b). Detailed substrate specificity was studied by using fucoidans from brown seaweeds as well as synthetic fucooligosaccharide with distinct structures. The fucoidanase FFA2 catalyzes the cleavage of $(1\rightarrow4)$ - α -glycosidic bonds in the fucoidan from *Fucus evanescens* within a structural fragment $(\rightarrow3)-\alpha\text{-L-Fucp}2\text{S}-(1\rightarrow4)-\alpha\text{-L-Fucp}2\text{S}-(1\rightarrow)n$ but not in a fragment $(\rightarrow3)-\alpha\text{-L-Fucp}2\text{S},4\text{S}-(1\rightarrow4)-\alpha\text{-L-Fucp}2\text{S}-(1\rightarrow)n$. The difference in substrate specificity and in the rate of enzymatic selectivity was investigated using synthetic di-, tetra- and octasaccharides built up of the alternative $(1\rightarrow4)$ - and $(1\rightarrow3)$ -linked $\alpha\text{-L-Fucp}2\text{S}$ units. Nonsulfated and

persulfated synthetic oligosaccharides were not transformed by the enzyme. Therefore FFA2 was specified as a poly [(1→4)- α -L-fucoside-2-sulfate] glycanohydrolase

Information on the enzymes involved in the degradation of fucoidans is extremely limited, and only the above mentioned descriptions of characterized bacterial enzymes have been published. To date, fucoidanases, where the sequence has not yet been determined, from bacteria, sea cucumber, sea urchin and marine fungi have been studied, their physicochemical characteristics have been determined, but only for a few of them has the type of action and specificity been established (Table 1.3). One of the main difficulties in the study of fucoidanases is the absence of a simple and sensitive method for detecting their catalytic activity. All currently available methods for establishing the hydrolytic activity of enzymes are of little use when analyzing fucoidanases.

Table 1.6 The biochemical properties of fucoidanases from natural organisms

Sources	Type of action	T-opt, °C	pH Opt	pH-stability	Inhibitors	Activators	Size, kDa
Bacteria: <i>Vibrio</i> sp. No.5 (Furukawa et al. 1992)	exo	38-45 38-45 38-45	6.0 6.0 7.5	4.0-9.0 4.0-9.0 4.0-9.0	Hg ²⁺ , Fe ³⁺ , Ag ⁺	Co ²⁺ 2mM	39.5 68.0 68.0
<i>P. citrea</i> KMM 3296, KMM 3297, KMM 3298 (Bakunina et al. 2002)	endo	n.d	6.5-7.0	n.d	n.d	n.d	n.d
<i>S. paucimobilis</i> PF-1 (Woo et al., 2015)	endo	n.d	n.d	n.d	n.d	n.d	n.d
<i>Flavobacterium</i> sp. SA-0082 (Sakai et al. 2003)	endo	40	7.5	n.d	Ag ⁺ , Zn ²⁺ , Cu ²⁺	0.4M NaCl	n.d
<i>Alteramonas</i> sp. SN-1009 (Sakai et al. 2004)	endo	30-35	6.5-8.0	n.d	Cu ²⁺ , Zn ²⁺	0.4M NaCl, Ca ²⁺	100
<i>M. fucanivorans</i> SW5 (Descamps et al. 2006)	endo	20-25	7.5	n.d	n.d	Ca ²⁺	105
<i>Formosa algae</i> KMM 3553 (Silchenko et al., 2013)	endo		6.5-9.1		Cu ²⁺	0.250mM Ca ²⁺ , Ba ²⁺ and Mg ²⁺	
Fungi <i>D. arenaria</i> TM94 (Wu et al. 2011b)	endo	50	6.0	5.0-7.0	n.d	n.d	180
<i>Fusarium</i> LD8 (Wu et al. 2011a)	endo	60	6.0	6.0	n.d	n.d	64
Invertebrates <i>M. yessoensis</i>	endo	n.d	5.5	n.d	n.d	n.d	85
<i>Haliotus</i> sp (Thanassi and Nakada, 1967)	endo	38	5.4	2.0-10	Hg ²⁺ , Mn ²⁺ , Ag ⁺ (0.001 M)	Mg ²⁺ (0.01M)	100-200
<i>S. nudus</i> (Sasaki et al. 1996)	exo	45	3.0-4.0	2.0-5.0	n.d	n.d	130
<i>L. sitkana</i> (Bilan et al. 2005)	endo	n.d	5.4	n.d	n.d	0.2M NaCl	n.d
	endo	n.d	8.5	n.d	0.2M NaCl	n.d	n.d
<i>Lambis</i> sp. (Silchenko et al. 2013)	endo	37	4.9	n.d	Cu ²⁺ , Zn ²⁺ , Hg ²⁺	Ca ²⁺ , Mg ²⁺ , Ba ²⁺	50

Nd: not determined

1.5.2.5. Determining fucoidanase activity

Reducing sugar assay

The activity of enzymes that hydrolyze polysaccharides is usually determined from a conventional reducing sugar assay which measures mono- and oligosaccharides produced during hydrolysis (Kusaykin et al., 2006). The amount of reducing sugars can be determined spectrophotometrically by reaction with dinitrosalicylic acid, the bicinchonite method, the ferricyanide method, or by the widely used Nelson-Somogy method. However, the colorimetric methods are of no use when the enzymes cleave only a small number of internal O-glycosidic bonds. Use of the reducing sugar assay has therefore been unsuccessful as previously mentioned (Colin et al., 2006). Even when fucoidan was efficiently cleaved, as clearly shown by electrophoresis, the reducing ability of the solution measured by the colorimetric method of Nelson was close to zero (Colin et al., 2006).

Viscosimetry

Because of the high molecular weight and viscosity of fucoidan, one of the methods used to establish the activity of the endo-type of action, by splitting internal bonds in the polysaccharide molecule, is viscosimetry. This method has sufficient sensitivity because a decrease in the viscosity of the substrate solution occurs after the disruption of several bonds in the macromolecule, when the formation of terminal groups cannot be determined by other methods. One of the advantages of viscosimetry is the possibility of quantitative evaluation of the obtained data. The disadvantages of the method, in the case of fucoidans, include the high concentration of substrate necessary for the preparation of viscous solutions, and a large volume of samples.

Electrophoresis of poly- and oligosaccharides

Electrophoresis is an effective way of analyzing carbohydrate-containing compounds. Methods of analytical electrophoretic separation include agarose gel electrophoresis, polyacrylamide gel electrophoresis, capillary electrophoresis, electrophoresis on cellulose acetate and nitrocellulose membrane, and electrophoresis of fluorescently labeled carbohydrates. These techniques are actively used in studying the structure of glycosaminoglycans (GAG) as well as in the study of glycoside hydrolases and lyases that transform polyanionic polysaccharides. However, most of these methods have not been used to study enzymes that can cleave fucoidans. The most frequently used methods of electrophoresis will be discussed below.

The FACE (Fluorophore-assisted carbohydrate electrophoresis) method

This is the modified version of PAGE. With FACE, monosaccharides and low-molecular

oligosaccharides are subjected to electrophoretic separation in which a compound with its own fluorescence is covalently attached by chemical methods to result in the formation of fluorescently-labeled derivatives. The labels can be either charged or uncharged fluorophores, such as 8-aminonaphthalene-1,3,6-trisulfonate sodium (ANTS) or 2-aminoacridone (2-AMAC). There is an electrophoretic separation of both negatively charged oligosaccharides and oligosaccharides that do not have their own charges but have received a charge due to fluorescent labels. Advantages of the method are extremely high sensitivity and good resolving power that allow a quantitative assessment of the content of low molecular weight products of chemical or enzymatic depolymerization of polysaccharides.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis has been used to characterize plant cell wall polysaccharide such as hemicellulose, pectins (Goubet et al., 2002), enzymatic hydrolysis products of carrageenan (Zablackis and Perez, 1990) and fucoidan (Descamps et al., 2006), (Silchenko et al. 2017). The electrophoresis of carbohydrates is often called Carbohydrate Polyacrylamide Gel Electrophoresis (C-PAGE) and is the most representative method for fucoidanase right now (Kusaykin et al., 2015), even though it is expensive and time-consuming. Most investigators have used this method with a few minor modifications. This method is quite sensitive and estimates the distribution of the sulfated fucooligosaccharides formed in the polyacrylamide gel depending on their molecular masses and charges (Silchenko et al. 2017). However, currently there is no commercially available standard of sulphated fucooligosaccharide and the specific structures of the oligosaccharides cannot yet be determined from the C-PAGE alone.

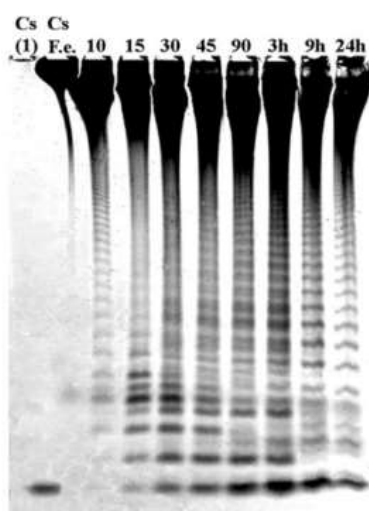


Figure 1.10 C-PAGE of kinetics of hydrolysis of fucoidan from *F. evanescens* by the recombinant fucoidanase FFA2 (Silchenko et al. 2017)

Agarose electrophoresis

This method enables the molecular weight and/or electron density of polyanionic polysaccharides to be estimated when necessary standards are available (Bilan et al., 2005). Electrophoresis in 0.6% agarose gel was used by Bilan et al. to confirm the action of fucoidanase from the marine mollusk *Littorina sitkana* (*Littorina kurila*) on fucoidan from *F. distichus*.

Turbidimetry

This method has been adapted for the analysis of fucoidanase activity of bacterial strains (Colin et al., 2006). To determine fucoidanase activity, the microorganisms were cultured in liquid medium with fucoidan. Aliquots of culture medium were collected after 1-7 days of growth and acidified solution of bovine serum albumin (BSA) was added. Insoluble complexes formed between BSA and high molecular weight fucoidan but not between BSA and fuco-oligosaccharides. The activity of the fucoidanases was evaluated by ability to reduce turbidity of the culture mixture.

Size exclusion chromatography (SEC)

Native fucoidan has high molecular weight and fucoidanase will produce oligo products with different sizes. So size exclusion chromatography (SEC) is also one of methods to detect oligosaccharides. However, until now there are no commercially available sulfated fucoidan oligosaccharides and therefore dextran sulfates of different sizes are used as standards (Silchenko et al. 2017).

Fucoidan agar plates

The cetavlon method is based on the ability of the cationic detergent hexadecyltrimethylammonium bromide (cetavlon) to form a white colored water-insoluble salt in the presence of fucoidan. as in Fig 1.11 (Silchenko et al., 2015). The fucoidan that is enzymatically degraded has a low charge density and is not precipitated with cetavlon. Transparent areas appear under colonies possessing fucoidanase activity. In Fig 1.11, note that the concentration of fucoidan in the medium (0.5%) was optimal. Fucoidan at lower concentrations (from 0.1% to 0.4%) gives a poor precipitate with cetavlon and hence any clearing is not visible.

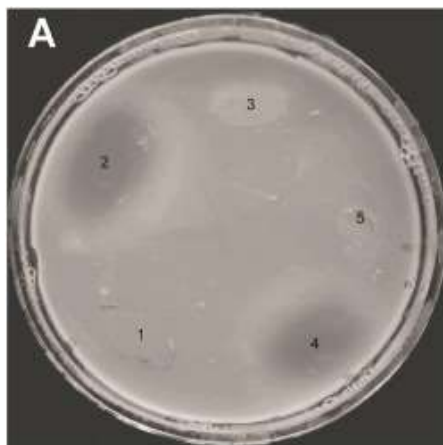


Figure 1.11 Cetavlon verification of fucoidanase activity. Solid medium with 0.5% fucoidans treated with a 1% aqueous solution of Cetavlon. 1, 3 and 5 are strains of MF 4-5, *Cytophaga* sp. ZBS 33F and *Coheasibacter* sp. SF 2-8 not producing fucoidanases; 2- *Formosa algae* KMM 3553T; 4 – *Formosa* sp. MF 2-3 are fucoidanase-producing strains (Silchenko et al., 2015).

Cetavlon is specific to the anionic groups of fucoidan (sulfate group), so the transparent areas that appear in fucoidan plates indicate not only fucoidanase but also sulfatase activity. Sulfatase plays a key role in the catabolism of various sulfated polysaccharides of marine origin (ulvans, carrageenans, agarans, fucoidans etc.). Desulfation of sulfated polysaccharides with sulfatases greatly simplifies and facilitates structural studies of such complex molecules of fucoidan. However, reports about true fucoidan sulfatases are rare. In the next section, information of marine polysaccharide sulfatases is summarized.

1.5.3. Sulfatases

In marine ecology, sulfated polysaccharides with complicated structures such as agar, carrageenans and fucoidan are carbon and energy sources for marine organisms. To break down complex polysaccharides, marine organisms secrete specific glycoside hydrolases (GHs), referred to as agarases, carrageenases and fucoidanases, which catalyze the hydrolysis of the glycosidic bonds in the backbone of their respective substrates. However, these enzymes are not sufficient alone to lead to the complete substrate assimilation. As revealed by the increasing number of sequenced marine microbial genomes, marine organisms possess a large number of sulfatases. Though the precise function of these sulfatases has not yet been elucidated, it is likely that they play an important role in the degradation of algal sulfated polysaccharides (Glöckner et al., 2003).

1.5.3.1 Sources and function of sulfatases

Sulfatases comprise a diverse family of enzymes (Parenti et al. 1997), (Hanson et al. 2004). Depending on the particular enzyme, the catalytic mechanism for sulfate ester hydrolysis proceeds by cleavage of either the S–O or the C–O bond, to yield in both cases inorganic sulfate (Fig 1.12)

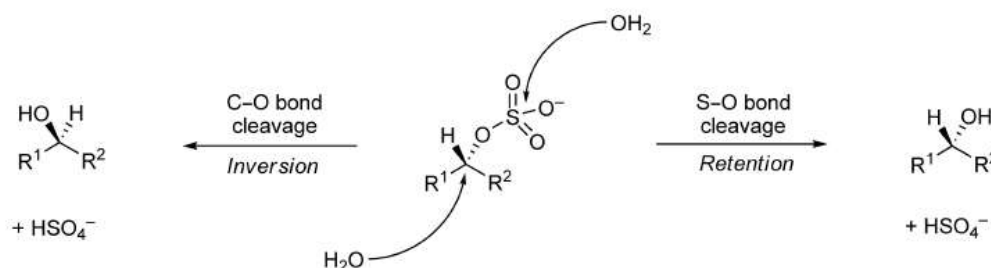


Figure 1.12 The catalytic mechanism of sulfate ester hydrolysis

Sulfatase enzymes have been found in most organisms from bacteria such as *Klebsiella* (Miech et al., 1998), (Henderson and Milazzo, 1979), *Enterobacter*, *Serratia*, *Pseudomonas* (Beil et al., 1995) and *Escherichia coli* (Benjdia et al., 2007) to humans that have been studied the most (Ghosh, 2007).

Sulfatases are involved in several processes integral to human health and disease (Hanson et al., 2004). Sulfatases have been implicated in hormone regulation, gamete interactions, and bone and cartilage formation (Reed et al., 2005) (Diez-Roux & Ballabio, 2005). High levels of sulfatase activity have been found in several adenomas (Matusiewicz et al., 2008), including prostate and breast cancers (Suzuki et al., 2011). Mammal sulfatases are involved in transformations of sulfated substrates such as mucopolysaccharides, sulfolipids and steroidal hormones (Diez-Roux & Ballabio, 2005).

Because sulfur like carbon, oxygen, and nitrogen is one of the most abundant elements in living organisms, sulfatases also play important roles in the cycling of sulfur in the environment. The principal roles of bacterial sulfatases are apparently to remove sulfate groups from sulfated molecules that can be used as carbon source. Several marine animals that feed on algae are known to secrete carbohydrate sulfatases as digestive enzymes, which cleave the sulfate ester bonds in dietary polysaccharides to improve digestion and absorption of marine polysaccharides (Hoshi & Moriya, 1980).

1.5.3.2. Nomenclature and classification of sulfatases

Sulfatases catalyze the hydrolysis of a diverse range of sulfate ester substrates, including: hydrophobic glucosinolates, steroids and thyroxine sulfates; amphiphilic sulfated carbohydrates found in glycosaminoglycans (GAGs), proteoglycans, and glycolipids; and water-soluble mono- and disaccharide sulfates. Based on the substrates of the sulfatases, they are classified in one of three major sulfatase groups: arylsulfatases (Nino et al., 2008), sulfur dioxygenases (Suzuki & Silver, 1966) and alkylsulfatases (Long et al., 2011). However, the number of sulfatases whose substrate has been characterized is limited in comparison to the huge diversity of sulfated compounds in nature, and this lack of information makes functional annotations of sulfatases particularly prone to flaws and misunderstanding.

SulfAtlas is a classification database which is based on sequence homology (<http://abims.sbr-roscoff.fr/sulfatlas/>). According to this database, it is possible to distinguish four families of sulfatases:

Family S1: The FGly-sulfatases include the vast majority of sulfatases. They catalyze the removal of sulfate ester groups through a hydrolytic mechanism (EC 3.1.6.- sulfuric ester hydrolases; EC 3.10.1.- sulfamidases). Family S1 sulfatase contains a unique catalytic residue, C α -formylglycine (FGly), which is post translationally generated from a conserved cysteine or serine. The post translational modification occurs when the polypeptide chain is still unfolded (Schmidt et al., 1995), (Miech et al., 1998)

Family S2: The alkylsulfodioxygenases such as alkylsulfatase AtsK from *Pseudomonas putida* S-313 (Kahnert & Kertesz, 2000). Family S2 sulfatases belong to the non-heme iron (II) alpha-ketoglutarate-dependent dioxygenase superfamily. They catalyze the oxygenolytic cleavage of a variety of different alkyl sulfate esters to the corresponding aldehyde and sulfate.

Family S3: Currently family S3 contains only alkyl sulfatases. The two characterized family S3 enzymes, the alkylsulfatase sdsA1 from *Pseudomonas aeruginosa* PAO1 (Hagelueken et al., 2006) and Pisa1 from *Pseudomonas sp.* DSM6611 (Knaus et al., 2012), comprise three domains: the N-terminal catalytic domain which belongs to the metallo-beta-lactamase superfamily and binds two zinc ions as cofactors; a central dimerization domain; and a C-terminal domain with a hydrophobic groove. A conserved histidine near the sulfate-binding site acts as the general acid for crucial protonation of the sulfate leaving group.

Family S4: The arylsulfohydrolases, represented by the arylsulfatase AtsA from *Pseudoalteromonas carrageenovora* 9T (Barbeyron et al., 1995). Family S4 sulfatases display

conserved residues (notably the signature (T/S)HxHxD) which form the catalytic zinc-binding sites found in glyoxalases II and metallo-beta-lactamases. Thus, both families S3 and S4 belong to the metallo-beta-lactamase superfamily.

One sulfatase belonging to the super family of amido-hydrolases has been purified from the marine bacterium *Pseudoalteromonas carrageenovora*. Phylogenetic studies show that the *P. carrageenovora* sulfatase thus represents the first characterized member of a new sulfatase family. The C-terminal domain of this family has strong similarity to the superfamily of amidohydrolases, which highlights the still unexplored diversity of marine polysaccharide modifying enzymes (Kim et al., 2005) (Genicot et al., 2014).

Most marine polysaccharide sulfatases are enzymes which can remove sulfate groups from the carbohydrate backbone of marine sulfated polysaccharides (carrageenans, fucoidans, ulvans etc.) and belong to the S1 family (SulfAtlas classification) (Hanson et al., 2004).

1.5.3.3. Structure and mechanisms of arylsulfatases

The structures of four sulfatases have been solved to date by X-ray crystal-structure analysis: HARSA (2.1 Å) (Lukatela et al., 1998), HARSB (2.5 Å) (Bond et al., 1997), and HARSC (2.6 Å) (Hernandez et al., 2003) from humans, and PARS (1.3 Å) from the gram-negative bacterium *Pseudomonas aeruginosa* (Boltes et al., 2001) Fig 1.13 .

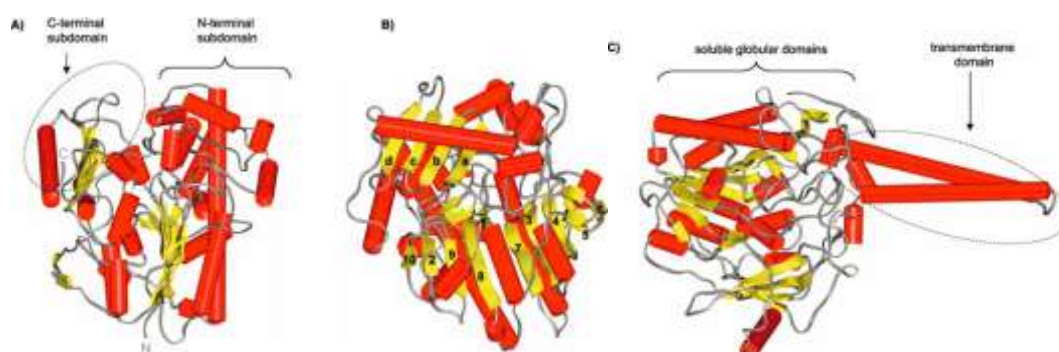


Figure 1.13 Crystal structures of different sulfatases (red cylinders: α helices, yellow arrows: β sheets). A) PARS, characterized by two subdomains with mixed α/β topology. B) Same structure of PARS, rotated 90°; the strands of the large β sheet within the N-terminal domain (numbered 1–10) and the small β sheet in the C-terminal domain (labeled a–d) are visible. C) HARSC, which shares high structural homology with PARS within the globular domains. This ER-resident protein also contains a transmembrane domain comprising two highly hydrophobic helices of 40 Å in length (Hanson et al., 2004)

Crystallographic and mutagenesis studies have provided valuable information about the active site of sulfatases (Fig 1.14).

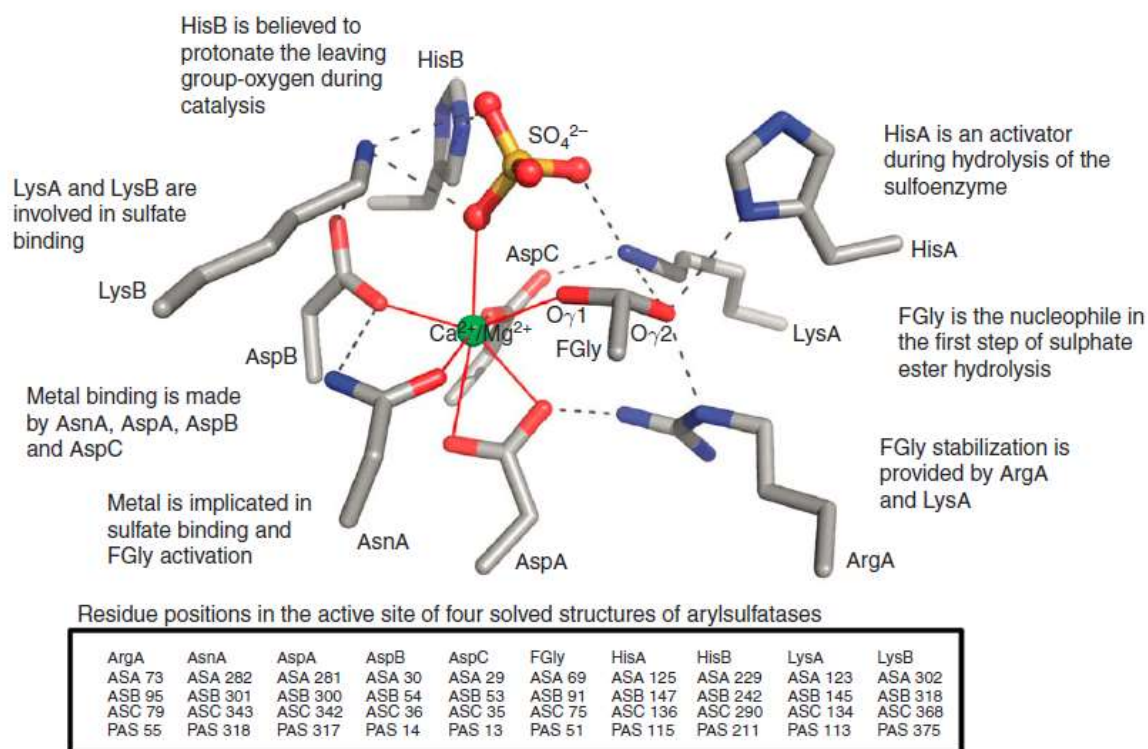


Figure 1.14 Sulfate anion interactions in the active site of the arylsulfatase PARS. The representation was drawn from the 1.3Å sulfate bound structure of the *P. aeuroginosa* AS (PARS, PDB code 1HDH). The numbering of residues is according to Hanson et al., 2004.

In each of the structures solved thus far the catalytic residues and geometry are conserved to a remarkable extent ($\text{rmsd} < 0.45 \text{ \AA}$), which supports the notion of a conserved mechanism for sulfate ester hydrolysis. The active site is comprised of 10 highly interconnected polar residues and a divalent metal cation; the positions and proposed functions of these components are summarized in Table 1.7.

The activity of arylsulfatases depends upon the posttranslational modification of highly conserved residues: cysteine in eukaryotes (Schmidt et al., 1995), (Selmer et al., 1996) and in some prokaryotes (Dierks et al., 1998), (Berteau et al., 2006) and serine in other prokaryotes (Miech et al., 1998). This posttranslational modification is enzymatically mediated and results in the oxidation of the cysteine or serine to yield an aldehyde residue, a formyl glycine (FGly) (Figura et al., 1998). The mechanism involving FGly and the key active-site residues based on the crystal structure of PARS and mutagenesis studies of HARS is represented in Fig 1.15.

Table 1.7 Positions and proposed functions of amino acids and metal cations in the active site
(Hanson et al., 2004)

Residue*	PARS	HARSA	HARSB	HARSC	Proposed function
FGly	51	69	91	75	Catalytic nucleophile as FGly hydrate
M	Ca ²⁺	Mg ²⁺	Ca ²⁺	Ca ²⁺	Substrate binding and activation. stabilization of FGly hydrate
AsnA	Asn318	Asn282	Asn301	Gln343	Metal coordination, activation of FGly hydrate
AspA	Asp317	Asp281	Asp300	Asp342	Metal coordination
AspB	Asp14	Asp30	Asp54	Asp36	Metal coordination
AspC	Asp13	Asp29	Asp63	Asp35	Metal coordination
ArgA	Arg55	Arg73	Arg95	Arg79	Stabilization of FGly hydrate
HisA	His115	His125	His 147	His136	Stabilization of FGly hydrate. elimination of sulfate hemiacetal FGS
HisB	His211	His229	His 242	His290	Substrate binding and activation. alcohol protonation
LysA	Lys 113	Lys 123	Lys 145	Lys134	Substrate binding and activation. stabilization of FGly hydrate
LysB	Lys 375	Lys 302	Lys 318	Lys368	Substrate binding and activation. alcohol protonation

*Generic residues are depicted schematically in Fig 1.14.

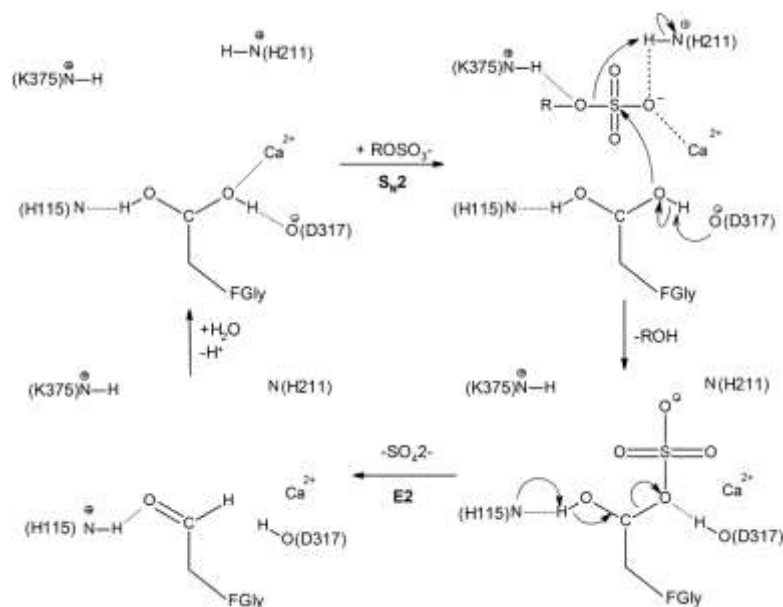


Figure 1.15 The catalytic mechanism involving FGly and key active-site residues based on the crystal structure of PARS and mutagenesis studies on HARS (Barbeyron et al., 2016)

1.5.3.4 The Post-translational FGly modification pathway

The arylsulfatases during activation undergo a unique post translational modification which leads to the conversion of an active site residue (serine or cysteine) into a C α -formylglycine; this modification of Cys or Ser side chains is essential for the activity of type I sulfatase (Fig 1.16). Formylglycine-generating enzyme (FGE) and anaerobic sulfatase-maturing enzyme (anSME) are the enzymes that produce FGly. FGE in particular is structurally and mechanistically distinct, and serves the sole function of activating type I sulfatase targets (Appel and Bertozzi, 2015).

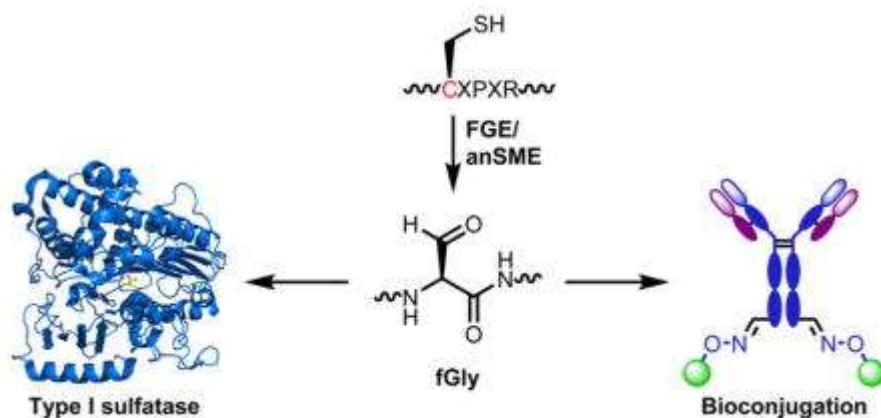


Figure 1.16 A modification of Cys or Ser side of type I sulfatase

Another pathway for producing FGly has been observed but the responsible enzyme(s) remain elusive. Recombinant expression of type I sulfatases in *E. coli* has been shown to produce active enzymes with varying proportions of FGly modification (Dierks et al., 1998). Analysis of the mature protein expressed in *E. coli* revealed the presence of formylglycine at the expected position, indicating that the cysteine is also converted to formylglycine in a prokaryotic sulfatase (Dierks et al., 1998). It has been reported that all the arylsulfatases characterized to date, except for the *Alteromonas* arylsulfatase, contain a conserved amino acid sequence motif in the active site in which cysteine or serine was modified to a formylglycine (FGly) residue (Kertesz, 2000). The *Pseudomonas* arylsulfatase contains a cysteine residue that is 100% converted to FGly (Dierks et al., 1998), while the *Klebsiella* sulfatase has only about 40% modified serine in the active site (Szameit et al., 1999).

Berteau and co-workers have shown that a putative *E. coli* anSME ortholog was unnecessary for the conversion of recombinant sulfatases, and that an unidentified O₂-dependent Cys-type fGly-generating enzyme must be responsible for installing the aldehyde. According to sequence data available at the time, as of 2007, approximately 10% of bacterial genomes were predicted to encode arylsulfatase genes without the co-occurrence of obvious FGE or anSME orthologs

(Benjdia et al., 2007). This is also true for several fungi, and even for the metazoan *Caenorhabditis elegans* which contains three predicted sulfatase encoding genes (Sardiello et al., 2005) (Landgrebe et al., 2003). *C. elegans* has been shown to utilize sulfatases for heparan sulfate remodeling, as also occurs in humans (Townley & Bülow, 2011).

In conclusion, these results reveal a new scenario for sulfatase maturation in which three systems are present (Fig 1.17): the oxygen-independent anSME, which can mature both Cys- and Ser-type sulfatases; the oxygen-dependent FGE system, specific for Cys-type sulfatases; and the newly proposed system responsible, as far as is currently known for the aerobic maturation of the Cys-type sulfatase in *E. coli* and which is probably present also in other bacteria.

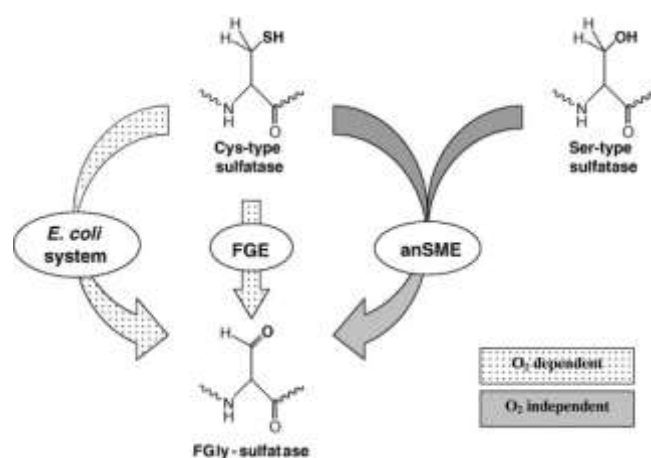


Figure 1.17 Maturation of sulfatases in prokaryotes. Anaerobic sulfatase maturing enzymes (anSME) are able to mature both types of sulfatases in an oxygen-independent manner, while two non-homologous systems, FGE and an unknown system present in *E. coli* (*E. coli* system), account for the specific maturation of Cys-type sulfatases in an oxygen-dependent manner (Benjdia et al., 2007).

1.5.3.4. Biochemically characterized marine polysaccharide sulfatases

Marine sulfated polysaccharides from seaweeds include agarans, carrageenans and fucoidans. Only a few marine polysaccharide sulfatases have been biochemically characterized and they almost attacked to agarans and carrageenans. These are all observed in marine bacteria (Table 1.8). There are very few reports of true fucoidan sulfatases. Although fucoidan sulfatase activity has been demonstrated in several bacterial strains and marine invertebrates, fucoidan sulfatase sequence is still unknown and no sulfatase genes are predicted in brown algal genome (Cock et al., 2010).

A sulfatase from the marine bacterium *Vibrio* sp. N-5 eliminates sulfate from sulfated fucose and short fucooligosaccharides released from fucoidan by fucoidanases. while activity towards the

native fucoidan polysaccharide was very limited (Furukawa et al. 1992a). A partially purified preparation from the bivalve mollusk *P. maximus* effectively desulfated a synthetic substrate p-nitrocatechol sulfate. C2 sulfated L-fucose and a natural fucoidan from *A. nodosum* of 13Kda. However, calculations have shown that only approximately 10% of the sulfate content was removed from the fucoidan sample containing 34% of sulfate substituent (Sasaki et al. 1996) (Daniel et al., 2001). A sulfatase hydrolyzing p-nitrophenylsulfate rather than natural fucoidan polysaccharides was found in the hepatopancreas of the marine mollusks *Haliotis sp.* and *L. kurila* (Kusaykin et al. 2006).

Table 1.8 Polysaccharide sulfatases from marine organisms

Organisms	Enzymes	MW (kDa)	Substrate	pH	Top/ T _{1/2}
<i>Thermotoga marina</i> NP_229503 (Lee et al., 2013)	arylsulfatase	65	p-nitrophenyl sulfate (pNPS) and agar	7.0	80
<i>Marinomonas</i> sp. FW-1 (Wang et al., 2015)	arylsulfatase	33	pNPS and agar	9.0	45
<i>Flammeovirga pacifica</i> (Gao et al., 2015)	arylsulfatase	56	pNPS and asparagus	8.0	40
<i>Patinopecten maximus</i> (Daniel et al., 2001)	Sulfatase	nd	pNCS, sulfated L-fucose, <i>A.nodosum</i> fucoidan	nd	nd
Marine mollusk <i>Littorina kurila</i> (Kusaykin et al., 2003)	Arylsulfatase	45	pNPS	5.4	20 min at 60°C
Marine mollusk <i>Turbo. sp</i> (Pesentseva et al., 2012)	Arylsulfatase	35	pNPS	7	15 min at 55°C
<i>Fusarium</i> sp. LE1 (Shvetsova et al., 2015)	Arylsulfatase	nd	pNPS	nd	nd
<i>P. carrageenovora</i> (Weigla and Yaphe 1966)	4O-κ-carrabiose sulfatase	55	κ-carrageenan	7.5	nd
<i>P. atlantica</i> T6c (Préchoux et al., 2013)	endo-ι-carrageenan-sulfatase	55.7	ι-carrageenan	7.5	35
<i>P. atlantica</i> T6c (Préchoux et al., 2013)	Psc ι-CgsA	115.9	ι-carrageenan	8.3	35-45
<i>P. carrageenovora</i> (Kim et al., 2005)	Arylsulfatase	35.8	agar	7.0-8.5	45
<i>Vibrio</i> sp. N-5 (Furukawa et al. 1992b)	Fucoidan-sulfatase	nd	Fucoidan	nd	nd

Nd – not identified

The degradation pathway of κ -carrageenan by *P. carrageenovora* PscT has been partially determined and involves a κ -carrageenase (Weigl & Yaphe, 1966) (Michel et al., 2001) to produce oligosaccharides which are readily degraded into neocarrabiose through the concerted action of a glycosulfatase (Weigl and Yaphe, 1966) and a neocarratetraose monosulfate hydrolase (McClean and Williamson, 1981). A similar pathway might be assumed for catabolism of ι -carrageenan because low ι -carrageenase activity has been detected in the crude extract of *P. carrageenovora* PscT (Henares et al., 2010).

However, iota-carrageenan sulfatase Psc ι -CgsA from *P. carrageenovora* PscT was reported to have an alternative mechanism in which ι -carrageenan is first desulfated and converted into α -carrageenan. The latter likely constitutes a metabolic intermediate that is probably subject to further degradation by an α -carrageenase and/or desulfation by other sulfatases, leading to the end-product of galactose residues. Except for the conversion of ι - into α -carrageenan, these steps are speculative and need to be corroborated (Genicot et al., 2014).

The pathway of fucoidan degradation is still unknown. A relationship between fucoidan-degrading enzymes and fucoidan sulfatase from *Vibrio* sp. N-5 was reported by (Furukawa et al. 1992a). In culture medium, fucoidanase and fucoidan sulfatase were induced by fucoidan.. Fucoidan sulfatase was induced with a lag time, whereas fucoidanase was induced rapidly. Thus the author presumed that fucoidan sulfatase might be induced by products degraded from fucoidan by fucoidanase.

Desulfated and deacetylated fucoidan from *F. evanescens* was hydrolyzed by fucoidanase from *Lambis* sp. more effectively than the original fucoidan. Thus the presence of sulfate and acetate groups in the molecule of fucoidan may interfere with enzyme hydrolysis. Removing the acetate and sulfate groups probably makes the glycosidic linkages more accessible to the action of the enzyme (Silchenko et al., 2014). In contrast, fucoidanase isolated from *Formosa algae* catalyzed the hydrolysis of fucoidans from *F. evanescens*, but desulfated fucoidan from *F. evanescens* was hydrolyzed very weakly (Silchenko et al., 2013). It might be the fucoidanase from *F. algae* is specific for the fucoidan structure. This point were confirmed by recombinant fucoidanase FFA2 from *F. algae*: this fucoidan catalyzed the cleavage of (1 \rightarrow 4)- α -glycosidic bonds in the fucoidan from *F. evanescens* within a structural fragment (\rightarrow 3)- α -L-Fucp2S-(1 \rightarrow 4)- α -L-Fucp2S-(1 \rightarrow)n but not in a fragment (\rightarrow 3)- α -L-Fucp2S,4S-(1 \rightarrow 4)- α -L-Fucp2S-(1 \rightarrow)n (Silchenko et al. 2017).

1.6. Conclusions

Fucoidans have a complex structure, so the sources and origin of the fucoidan always has to be specified. In this review we also see that the fucoidan modifying enzymes are widespread in sea organisms. Thus, given this great diversity of fucoidans, the prospects of discovering fucoidan modifying enzymes in marine environments with various specificities is very high. A few enzymes have been found which exhibit fucoidan-modifying characteristics. They were reported to cleave the core or fundamental linkages in the backbone of fucoidan $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ -L-fucose residues. The application of these enzymes may thus allow elucidation of the structure of the fucoidans and may help provide further insight into the biological activity of fucoidans isolated from various sources.

Chapter 2. Isolation and screening of aerobic marine bacteria from sea cucumber to identify novel fucoidan modifying enzymes

Fucoidans - sulfated polysaccharides - are a rich carbon source for different organisms, and are expected to be degraded by these organisms, such as invertebrates, bacteria and fungi. Marine microorganisms that hydrolyze fucoidan are of interest for their potential for facilitating the utilization of fucoidan in the food, cosmetics or pharmaceutical industries. For these reasons, screening for fucoidan modifying enzymes (fucoidanase and sulfatase) was primarily carried out on sea cucumber gut bacteria.

The objectives of the work were the following:

- To prepare fucoidan from *S. mcclurei* and *T. ornata* collected in Nhatrang Bay, Vietnam.
- To isolate aerobic marine bacteria from gut of sea cucumbers co-existing with *S. mcclurei* and *T. ornata* brown seaweeds.
- To screen all isolated strains production of fucoidan-degrading enzymes by the fucoidan-agar plate method; the potential strains were identified by 16S rDNA.
- To confirm sulfatase activity by 5-Bromo-4-chloro-3-indolyl sulfate potassium salt (X-SO₄) plate screening, and endo-fucoidanase activity by the C-PAGE method, and alginate lyases by reducing sugar method.
- To analyze the sequences of potential strains to find the fucoidanase and sulfatase genes.'

The study was based on the hypothesis (1) stated on page 1 of this thesis.

This study is related to the manuscript: Isolation and screening of aerobic marine bacteria from sea cucumber to identify novel fucoidan modifying enzymes

2.1 Preparation and composition analysis of fucoidans

Fucoidan from Vietnamese brown seaweed *S. mcclurei* and *T. ornata* was extracted and the results described in Table 2.1. The results indicate that *S. mcclurei* and *T. ornata* fucoidans are sulfated galactose fucans, as expected. The main component of *S. mcclurei* fucoidan is fucose (38.5%) and galactose (32.3%) with up to 33% sulfate content while *T. ornata* fucoidan is fucose (55.8%) and galactose (24.8%) with 25.30% sulfate content. This crude fucoidan was used for screening marine bacteria for ability to produce fucoidan modifying enzymes for the screening step using the fucoidan-agar plate method.

Table 2.1 Monosaccharide composition of fucoidan from *S. mcclurei* and *T. ornata*

Fucoidan sources	Uronic acid (% w/w)	SO ₃ Na (% w/w)	Monosaccharide composition (% mol)				
			Fucose	Galactose	Glucose	Mannose	Xylose
<i>S. mcclurei</i> crude	17.87	33.15	38.5	33.1	20.6	4.2	3.6
<i>S. mcclurei</i> pure	-	35.03	58.5	41.5	-	-	-
<i>T. ornata</i>	7.50	25.30	55.8	24.8	1.2	9.2	9.0

(-) not detected

2.2 Isolation and screening of marine bacteria of fucoidan modifying enzymes

From fifteen sea cucumber samples that were collected in at different positions Nha Trang Bay, Vietnam, 97 strains (MB1-97) of aerobic bacteria were isolated. Isolated strains of bacteria have different colony characteristics, so we presumed that they might be different strains of bacteria. These strains are used to screen fucoidan modifying activities on fucoidan from *S. mcclurei* and *T. ornata* by fucoidan agar - plate method (Fig 2.1). The results showed that 15 strains created a clear zone in the agar plate suggesting substantial modifications to the fucoidan. MB87 showed the highest activity on pure fucoidan from both *S. mcclurei* and *T.ornata*.

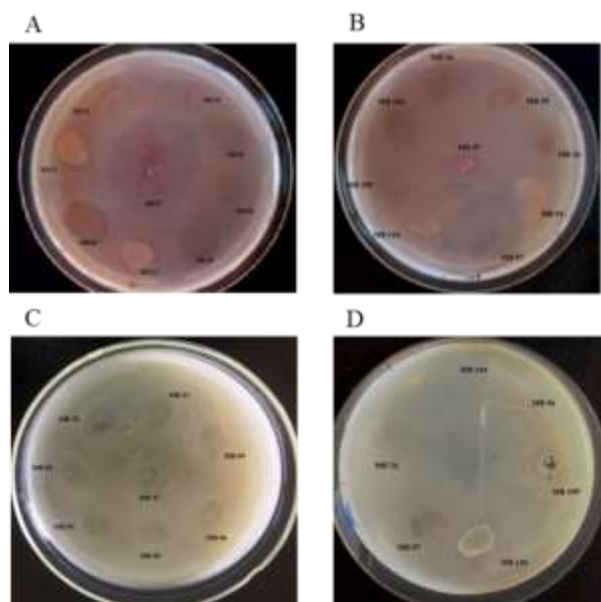


Figure 2.1 Fucoidan-agar plate method of detection of fucoidan-modifying enzymes from bacteria using solid medium. A) and B) fucoidan from *S. mcclurei*; C) and D) fucoidan from *T. ornata* fucoidan treated with a 1% aqueous solution of cetavlon.

The identification of 15 selected strains was conducted by 16S rDNA and the results are presented in Table 2.2.

Table 2.2 Fucoidan modifying activities of isolated marine bacteria

Code	Closest relatives (by 16S rDNA)	Fucoidan – plate method		Sulfatase
		<i>S. mcclurei</i> fucoidan	<i>T. ornata</i> fucoidan	X-SO ₄
MB 03	<i>Pseudomonas sp.</i>	+	+	-
MB 04	<i>Pseudomonas sp.</i>	+	+	-
MB 05	<i>Ancinobacter baumannii</i>	+	+	-
MB 06	<i>Pseudomonas sp.</i>	+	+	-
MB 09	<i>Ocyanomonas sp.</i>	+	+	-
MB 26	<i>Bacillus sp.</i>	+	+	-
MB 31	<i>Shawannella sp.</i>	+	+	-
MB 32	<i>Shawannella sp.</i>	+	+	-
MB 47	<i>Pseudoalteromonas sp.</i>	+	+	+++
MB 87	<i>Cobetia sp.</i>	+++	+	+
MB 95	<i>Bacillus aryabhattai</i>	+	+	-
MB 96	<i>Bacillus sp.</i>	+	-	-
MB 104	<i>Pseudoalteromonas sp.</i>	+	+	+
MB 109	<i>Photobactenia sp.</i>	+	-	-
MB 110	<i>Photobactenia sp.</i>	+	-	-

(nd) not detected

(-) no activity

(+) have activity

(+++) very high activity

Confirmation of fucoidan modifying ability

To confirm fucoidanase activity, the Carbohydrate - Polyacrylamide gel electrophoresis (C-PAGE) method was used. Two different pure fucoidan samples from *S. mcclurei* and *T. ornata* were incubated with the individual intracellular fraction of 15 selected marine bacterial strains. C-PAGE results showed that only *Cobetia sp.* MB87 displayed positive activity on fucoidan from both *S. mcclurei* and *T. ornata* (Fig. 2.2). However, the fucoidanase activity was quite low and was easily lost during extraction and purification from the bacterium, probably due to proteolytic activity. Thus our efforts to purify fucoidanases from *Cobetia sp.* MB87 were not successful.

The sulfatase and α -L-fucosidase activities were investigated on X-SO₄ and X-Fuc agar plates, respectively. However, no strains were found to be active on X-Fuc plates, suggesting that MB47 and MB87 do not produce fucosidases. *Pseudoalteromonas sp.* MB47, *Pseudoalteromonas* MB104 and *Cobetia sp.* MB87 bacterial strains gave a positive signal on X-SO₄ plates. These colonies turned blue after 1 to 3 days incubation at 30°C (Fig 2.3 A). *Pseudoalteromonas sp.* MB47 showed the strongest blue colonies after 1 day at 30°C. Colonies of *Pseudoalteromonas*

MB104 turn blue after three days and the colonies of *Cobetia* sp. MB87 started to become blue around their borders after three days and completely blue after five to seven days. These results suggest that these strains produced active sulfatases intracellularly, which was verified when testing the crude extracts (Fig 2.3 B). However, crude extracts from all three bacterial strains did not show activity on native fucoidan when measured by SO₄ release.



Figure 2.2 C-PAGE product profiles of fucoidan degradation using crude enzymes. (1) *S. mcclurei* fucoidan, (2) enzymatic reaction of intracellular fraction of MB87 and *S. mcclurei* fucoidan, (3) *T. ornata* fucoidan, (4) enzymatic reaction of intracellular fraction of MB87 and *S. mcclurei* fucoidan.

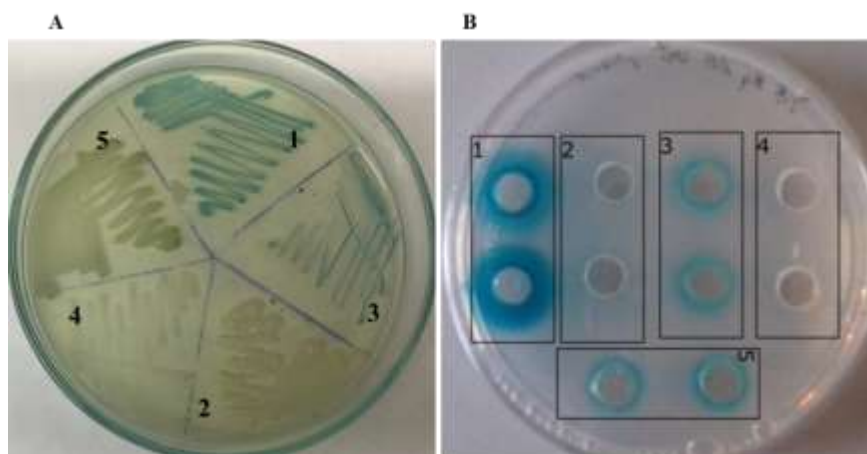


Figure 2.3 Sulfatase activity on X-SO₄ plate. A) *Pseudoalteromonas* sp. MB47 colonies; B1) crude extract from *Pseudoalteromonas* sp. MB47; B2) Crude extract from *Pseudoalteromonas* sp. MB06; B3) Crude extract from *Cobetia* sp. MB87; B4) Crude extracts from *Pseudoalteromonas* sp. MB04; B5) Crude extract from *Pseudoalteromonas* sp. MB104.

2.3. Sequence analysis of selected strains

After functional screening of bacteria isolated from sea cucumber gut, sequencing and draft genome assembly was performed on the selected isolates *Pseudoalteromonas* sp. MB47 and *Cobetia* sp. MB 87 (Table 2.3).

Table 2.3 The quality and completeness of the draft genome assemblies of selected marine isolates

Isolates	Completeness	Genomes size	No. contigs	GC	CDS	tRNA
<i>Pseudoalteromonas</i> sp MB47	100	4545585	31	41.1	4017	94
<i>Cobetia</i> sp MB 87	80.55	3101384	12	82.8	2581	67

To identify the putative fucoidanase-encoding enzymes, we aligned predicted protein sequences against two sequences of enzymes with known fucoidanase activity from the CAZy GH107 family. The matches identified within the genomes of *Pseudoalteromonas* sp. MB47 were limited to non-catalytic cadherin-like or immunoglobulin-like domains.

We also identified a number of carbohydrate-active enzymes in the analyzed genomes based on the HMMscan search against a stand-alone dbCAN database. The number of genes encoding putative CAZymes was 72 and 157 for MB87 and MB47, respectively (Table 2.4). The *Pseudoalteromonas* MB47 exhibited the highest diversity of encoded CAZymes. The draft genome of MB47 contained CAZymes with proposed activity on a number of complex carbohydrates, such as carrageenan or agar (GH16), pectin (PL1 and PL11), alginate (PL6, PL7 and PL17) and chitins (GH18). The draft genome of *Cobetia* sp. MB87 did not contain alginate lyases or fucoidanases. In addition, we also identified a number of sulfatases in the analyzed genomes. There were seven putative sulfatases identified from the genome of MB47, while only two were identified in the *Cobetia* sp. MB87 genome.

Table 2.4 Carbohydrate-active enzymes encoded in the genomes of selected marine bacteria

Isolates	CAZymes	Sulfatases	GH107 (Blast >10e ⁻⁷)	Alginate lyases			
				PL6	PL7	PL15	PL17
<i>Pseudoalteromonas</i> sp MB47	157	7	3	3	1	0	2
<i>Cobetia</i> sp MB 87	72	2	0	0	0	0	0

2.4. Discussion and conclusions

From gut of sea cucumbers collected in Nhatrang Bay, 97 marine bacteria were isolated. The sea cucumber samples co-existed with *S. mcclurei* and *T. ornata* brown seaweeds distributed the most this region (Ly and Hau, 2010). Hence, bacteria associated with these sea cucumber might produce enzymes that are able to modify fucoidan from *S. mcclurei* and *T. ornata*. In addition, crude fucoidan from *S. mcclurei* and *T. ornata* contains fucose, galactose, mannose, xylose, uronic acid and sulfate (Table 2.1), thus in order to modify these fucoidans, a complex of various enzymes (fucoidanases, sulfatases, fucosidases, galactosidase...etc) capable of transforming fucoidan needs to be produced by the bacteria. However, it is fucosidase and fucoidanase which are the enzymes that can attack the molecular backbone of fucoidan. Other enzymes, like galactosidase, attack the branches of fucoidan and would not change the molecular weight too much. Therefore, in our study we focused on finding fucosidase, fucoidanase, sulfatase and new enzymes that cleave the fucose-galactose linkages of the fucoidan molecule. The α -L-fucosidases that release L-fucose from the non-reducing end of fucoidan by X-Fuc plate were not found. Hence, the fucoidan-plate method (Silchenko et al. 2015) was the next step to find potential bacterial strains producing fucoidanase and sulfatase and possibly also new enzymes that cleave fucose-galactose linkages of the fucoidan molecule.

Fifteen marine bacterial strains produced a positive signal on fucoidan agar plates (Fig.2.1). These strains included *Pseudomonas* sp., *Ancinobacter baumannii*, *Oceanomonas* sp., *Bacillus* sp., *Shawannella* sp., *Photobactenia* sp and *Cobetia* sp. all belong to the γ -proteobacteria (Table 2.2). Our results agree with previous publications whose authors also reported that members of γ -proteobacteria were the predominant bacterial group in the gut contents of sea cucumbers and in the surrounding sediment (Gao et al. 2014). Unculturable γ -proteobacteria were predominant in gut contents of sea cucumbers cultured under the pond culture mode (Gao et al., 2010) by metagenomic discovery, and culturable γ -proteobacteria (*Vibrio*, *Pseudomonas*) were found to be dominant in the digestive tract of *A. japonicus* (Sun and Chen, 1989).

Our results also provide evidence that bacterial populations from sea cucumber gut can produce fucoidan-modifying enzymes according to our hypothesis. Production of fucoidanases has also previously been reported for γ -proteobacteria such as *Sphingomonas paucimobilis* PF-1 (Kim et al., 2015), *Alteromonas* sp. SN-1009 (Sakai et al., 2004), *Vibrio* sp. N-5 (Furukawa et al. 1992a) and *Pseudoalteromonas citrea* KMM 3296, KMM 3297, KMM 3298 (Bakunina et al., 2002), but

few sequences of fucoidanases from γ -proteobacteria have been reported (Fda1 and Fda2 from *Alteromonas* sp. SN-1009).

The intracellular crude extract from *Cobetia* sp. MB87 was shown to have activity on pure fucoidan from both *S. mcclurei* and *T. ornata* (Fig 2.2). This result suggests that *Cobetia* sp. MB87 utilizes fucoidan by degrading it and that the fucoidan-degrading enzyme was produced inside the cells. However, fucoidan has high molecular weight, so it is unclear how bacteria can absorb fucoidan molecules into the cells and then degrades them to low molecular weight, or whether the bacteria have another pathway to use fucoidan as food. We presume that the fucoidanase from *Cobetia* sp. MB87 was excreted out side the cells and quickly degraded under laboratory conditions, then led to no activity was seen from extracellular of *Cobetia* sp. MB87 in our experiment. There was still a little enzyme inside the cells so the activity was not high. Extracellular fucoidanase was also were found from *Alteromonas* sp. SN-1009 (Sakai et al., 2004) which belongs to γ -proteobacteria.

The genome sequence of *Cobetia* sp. MB87 was analyzed (Table 2.4) and no fucoidanase encoding genes were found. This might be because the *Cobetia* sp. MB87 belongs to the γ -proteobacteria while almost all of the eight known fucoidanase sequences in the CAZy database (Lombard et al., 2014) and FFA1 and FFA2 from the marine bacterium *Formosa algae* are from species belonging to the Bacteroidetes. Fda1, Fda2 and SVI are sequences from γ -proteobacteria but we did not find any sequences resembling these sequences in our bacterium (MB87). Therefore purification of a new fucoidanase from *Cobetia* sp. MB87 and determination of the encoding gene would extend our knowledge about fucoidanase encoding genes.

Pseudoalteromonas sp. MB47 and MB104 and *Cobetia* sp. MB87 seem to produce sulfatases (Fig 2.3). However, desulfation of fucoidan from *S. mcclurei* and *T. ornata* by crude enzymes of the two *Pseudoalteromonas* sp. species MB47 and MB104, as well as the *Cobetia* sp. MB87, was not successful using HPLC. This might be because the sulfatases are only capable of hydrolyzing small fucoidan oligosaccharides rather than the natural fucoidan polymers, and the fucoidanases had lost their activity before they had degraded the fucoidan into oligosaccharides on which the sulfatases could act. Genome analysis of *Pseudoalteromonas* species MB47 and *Cobetia* sp. MB87 showed seven and two putative sulfatases, respectively.

The cloning, expression and purification of sulfatases from *Pseudoalteromonas* species MB47 will be presented in the next chapter. We chose this strain because it exhibited the highest

activity on X-SO₄ substrate, and thus the chance of finding fucoidan sulfatase from seven sequences of this strain was higher than with the two sulfatase sequences from MB87.

Chapter 3: Fucoidan sulfatases: cloning, expression and biochemical characterization

Fucoidans are sulfated polysaccharides and *S. mcclurei* fucoidan has up to 35% of sulfate content. Sulfate groups are present on the fucose residues of the backbone. Sulfates are thought to affect the bioactivity of fucoidan as well as the activity of the fucoidanases. Thus, in the present work, our main aim was the discovery of new sulfatases able to desulfate fucoidan.

The objectives of the work were the following:

- To identify the putative sulfatase encoding genes from the genome of *Pseudoalteromonas* sp. MB47 which expressed the highest sulfatase activity?
- To express and purify the identified sulfatases by using a system that ensures high protein yield and high activity.
- To determine the optimum reaction conditions for enhanced sulfatase activity, such as divalent cations, pH and temperature, as well as to determine the kinetics of the selected sulfatases.
- Lastly, to use the selected sulfatase under optimal conditions to modify fucoidan.'

The study was investigated based on hypothesis (2) that stated on page 2 of this thesis.

The following will be related to the work of paper: A thermostable fucoidan active sulfatase (co-first author)

3.1. Identification of the putative sulfatase from *Pseudoalteromonas* sp. MB47

Five putative sulfatases, ARSUL1, ARSUL2, ARSF1, ARSUL3 and ARSUL4, from *Pseudoalteromonas* sp. MB 47 were predicted by sequence homology. ARSUL2 is 30% identical with ARS (previously AtsA; P51691) from *Pseudomonas aeruginosa*. ARS was reported to be active on p-nitrocatechol sulfate (pNCS) substrate and the crystal structure has also been solved (Beil et al., 1995), (Boltes et al., 2001). Thus we took ARS as the positive control for sulfatase activity on pNCS in our study. ARSUL1, ARSUL3 and ARSUL4 are 46%, 51% and 47% identical, respectively, with Ary423 from *Flammeovirga pacifica*, which is active on p-nitrophenyl sulfate (pNPS) and crude asparagus cell wall extracts (Gao et al., 2015). ARSF11595 is 38% identical with an *E. coli* sequence which has not yet been characterized. A hit called Ps3148 was found in the *Pseudoalteromonas* sp. genome, which is 85% identical with AER35705 from *Pseudoalteromonas carrageenovora* (Genicot et al., 2014). Thus Ary423 and Psc 1-CgsA were also

included in our study together with six potential sulfate modifying enzymes from *Pseudoalteromonas* sp. MB 47 (Table 3.1).

Table 3. 1. Sulfatase sources features and molecular weight

Enzyme name		Organism	Features ^a	bp	Length (aa) ^b	Expected molecular weight (kDa)
1	ARSUL1	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	1482	494	57kDa
2	ARSUL2	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	2271	757	90kDa
3	ARSF1	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	1521	507	57kDa
4	ARSUL3	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	1440	480	55kDa
5	Ps3148	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	3117	1039	116kDa
6	ARSUL4	<i>Pseudoalteromonas</i> sp.	His ₆ (N-term)	1467	489	56kDa
7	ARS-P51691	<i>Pseudomonas aeruginosa</i> PAO1	His ₆ (N-term)	1611	537	61kDa
8	Ary432 - AKL72071	<i>Flammeovirga pacifica</i>	His ₆ (N-term)		511	56kDa
9	AER35705	<i>Pseudoalteromonas carrageenovora</i>	His ₆ (N-term)		1033	117kDa

^a Wild type signal peptide had been removed for codon-optimized synthesized construct.

^b Including his-tags

3.2 Expression and purification of the identified sulfatases

Nine enzymes presented in Table 3.1 were cloned and expressed in *E. coli* BL2 (DE3). The cells and the intracellular crude extracts were streaked on LB plates containing 5-Bromo-4-chloro-3-indolyl sulfate potassium salt (X-SO₄) for screening sulfatase activity at 37°C. The *E. coli* cells with sulfatases ARS and ARSF1 produced blue colonies, suggesting that the sulfatases were expressed and active on X-SO₄ (Fig 3.1 A). The intracellular crude extracts of ARS and ARSF1 also showed sulfatase activity (Fig 3.1 B). ARSF1 was selected for further experimentation.

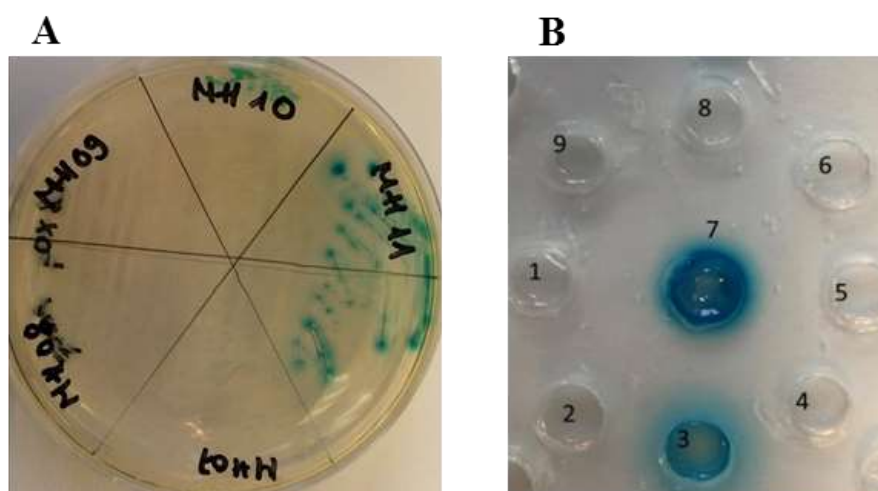


Figure 3.1 Screening sulfatase activity using X-SO₄ plates. A) Expression strains of *E.coli* BL21 (DE3) harboring ARSF1 (MH07), ARSUL3 (MH 08), AER35705 (MH09), Ps3148, ARS (MH10) and ARS-P51691 (MH11) on X-SO₄ plate. ARS (MH11) turned blue after 24h at 37°C. B) Crude extracts 1) ARSUL1, 2) ARSUL2, 3) ARSF1, 4) ARSUL3, 5) Ps3148, 6) ARSUL4, 7) ARS, 8) Ary432 and 9) ARE 35705.

ARSF1 was expressed in different *E. coli* strains by IPTG induction. The expression hosts used were BL21 (DE3), BL21 (DE3) with the pGro7 chaperone, BL21 (DE3) PlysS, C41 (DE3). Sulfatase activity was tested on *E. coli* lysates after sonication on plates containing X-SO₄ substrate (Fig 3.2).



Figure 3.2 ARSF1 from different expression *E.coli* strains exhibiting activity on X-SO₄ plates. BL21 (DE3) (007), BL21 (DE3) with the pGro7 chaperone (19), BL21 (DE3)PlysS (36), C41 (DE3) (43)

BL21 (DE3) with the pGro7 chaperone was the optimal expression strain and was selected to produce enzyme. The ARSF1 sulfatase was purified and desalted. The purified protein was very pure and gave the expected molecular weight of approximately 57 kDa (Fig 3.3). The yield of ARSF1 was surprisingly high, up to 57% of the total protein in the *E. coli* lysate.

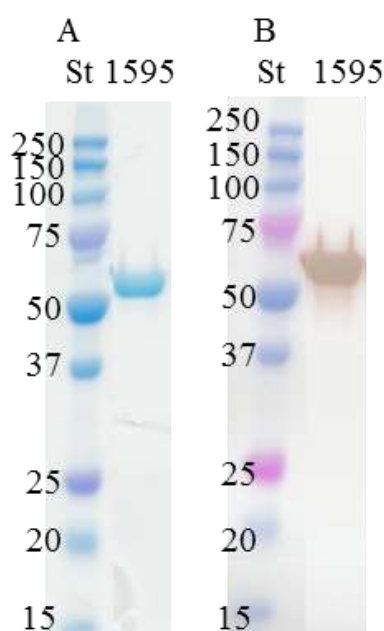


Figure 3.3 Purified recombinantly expressed ARSF1 sulfatase. A) SDS-PAGE. B) Western blots of (St) protein plus molecular weight marker. The expected molecular weight of the recombinant sulfatase ARSF1 was 57 kDa.

3.4 Characterization of the recombinant ARSF1 sulfatase

The recombinant sulfatase ARSF1 was further investigated with regards to 3D structure as well as reaction conditions for optimal enzyme activity *in vitro*. These parameters included divalent metal ion dependence, pH, temperature optimum, thermostability as well as K_m and V_{max} on p-nitro catechol sulfate (pNCS) substrate. Sulfatase activity was routinely assayed with pNCS as substrate using spectrophotometric determination of p-nitro catechol (pNCS) at 515nm (John, 1995).

3.4.1. 3D structure modelling of ARSF1

The 3D homology model of ARSF1 was inspected visually in PyMOL and its quality was checked using the QMEAN4 Z-score. The model showed a dimeric structure and the expected β -sandwich fold (Fig 3.4). In both monomers a calcium ion was bound in the active sites H281, N338 and K354. Thus in the next experiment we characterized the effect of divalent cations, including Ca^{2+} , on ARSF1.

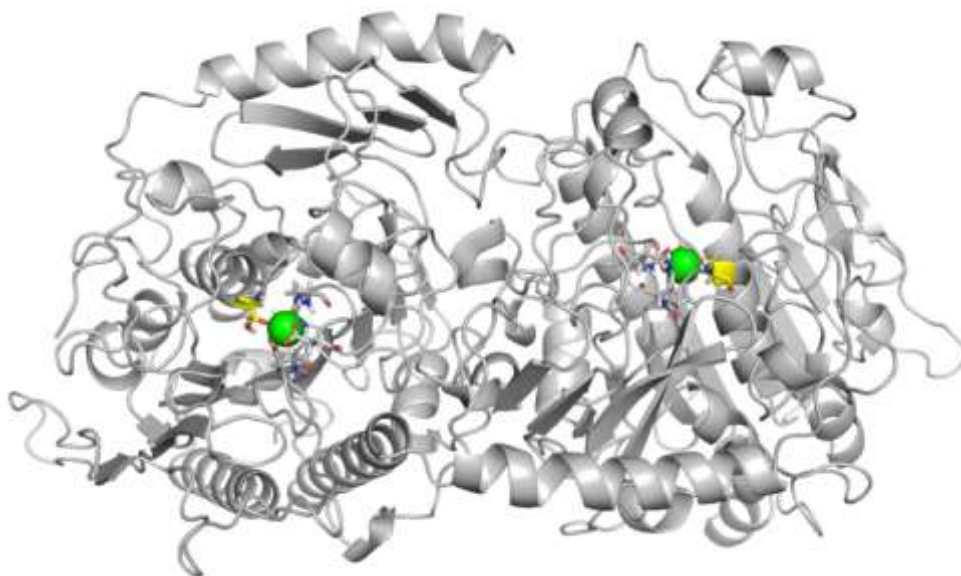


Figure 3.4 3D homology model of ARSF1. The 3D structure shows a beta-sandwich fold. The structure is predicted as a homodimer and a calcium ion (green sphere) is predicted in each active site. The calcium coordinating amino acid residues including the active site formyl glycine (yellow sticks) are shown as grey sticks.

3.4.2. The effect of divalent cations on ARSF1

The influence of divalent cations on ARSF1 activity was determined after ARSF1 sulfatase was pre-incubated with 0-10 mM CaCl_2 , MgCl_2 , MnCl_2 , NiCl_2 , CuCl_2 , ZnCl_2 and FeCl_2 for 5 min, and the activity in each case on pNCS was subsequently measured under standard conditions (37°C, 60 mins; Table 3.2).

Table 3.2 The effect of divalent cations on ARSF1 activity

Divalent cations	Relative activity (%)			
	0mM	2mM	5mM	10mM
Ca^{2+}	100	258	452	608
Mg^{2+}	100	110	124	127
Mn^{2+}	100	131	nd	nd
Ni^{2+}	100	16	15	nd
Zn^{2+}	100	6	nd	nd
Cu^{2+}	100	35	nd	nd
Fe^{2+}	100	8	nd	nd

nd: not determined.

100% relative activity of ARSF1 at 37°C app. 0.3 U/mg protein

The results showed that the sulfatase activity increased app. six folds with addition of 10mM Ca^{2+} . 2mM Mg^{2+} and Mn^{2+} increased sulfatase activity by 110% and 131%, respectively. The heavy

metals Ni^{2+} , Zn^{2+} , Cu^{2+} and Fe^{2+} at 2mM inhibited ARSF1 sulfatase activity by 16%, 6%, 35% and 8%, respectively.

3.4.3. Optimal pH and temperate conditions

The effect of pH on sulfatase activity was assessed as a function of catalytic efficiency by measuring kinetic parameters in two overlapping pH buffer systems – 200mM acetate buffer at pH 5.0 and 200mM Tris-HCl buffer at pH 6.5 and 8.0 – to span the range pH 5.0 to 8.0. The temperature dependence of ARSF1 activity was monitored by performing incubations with pNCS at 30, 45, 60, 60 and 75°C (at pH 6.5) in a reaction mixture containing 10mM CaCl_2 and 125mM NaCl. The results showed that the optimal pH of ARSF1 activity on pNCS was 6.5 and the optimal temperature was 68°C (Fig 3.5)

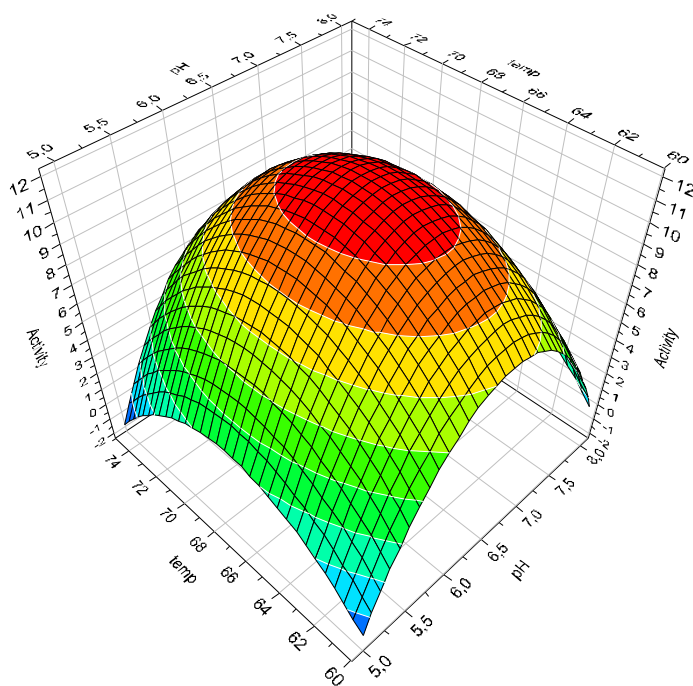


Figure 3.5 surface responses as a function of temperature and pH on ARSF1 sulfatase activity. The incubation mixture contained 10mM Ca^{2+} . The curve in the plot is isorates (in all the points of the value of activity is the same). The color varies from blue (low sulfatase activity) to red (high sulfatase activity).

For desulfated fucoidan, we had only native fucoidan from *S. mcclurei*, *F. evanescens* but we did not have the sulfated oligofucoidan standard. Thus we had to create oligofucoidan products by fucoidanase and more details of procedures are given in the next chapter. And the conditions for fucoidanase reaction also required 250 mM NaCl. Hence, we also identified the effect of NaCl on sulfatase.

3.4.3. The effect of NaCl concentration on sulfatase activity

The effect of NaCl concentration on sulfatase activity was assessed using 0, 62.5 and 125 mM NaCl for 0 to 50 mins (Fig 3.6). The results showed that sulfatase activity of ARSF1 at 0, 62.5 and 125mM NaCl was 19 ± 0.2 U, 18 ± 1.2 U and 14 ± 0.6 U, respectively.

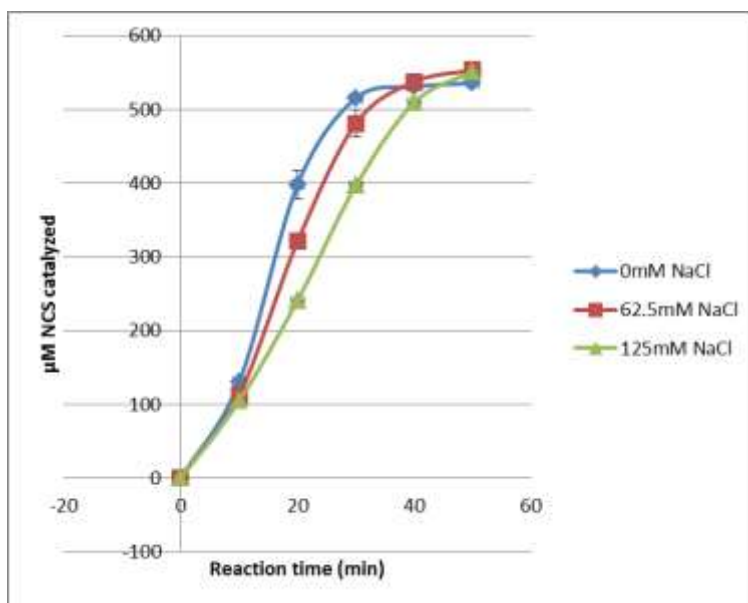


Figure 3.6 The effect of NaCl on sulfatase activity. Desulfation of NCS by the sulfatase showed that the maximum activity was reached within 30min in the absence of NaCl, while at 62.5mM and 125mM NaCl the maximum was reached in approximately 50 min or 60 min, respectively.

Fucoidans are polysaccharides with a large molecular mass and complex structure and therefore the sulfatase might need a longer time to find its target and cleave SO_4 groups of the fucoidan molecule. The optimal temperature of ARSF1 was shown to be very high at 68°C , so it was also necessary to determine the thermal stability of ARSF1.

3.4.4. The thermal stability of ARSF1

The thermal stability of the recombinant ARSF1 was evaluated by measuring the residual enzyme activity after pre-incubation of the enzyme in 40mM Tris-HCl, 10mM CaCl_2 , and at pH 6.5 and 68°C for various times (1h, 2h, 3h, 4h, 5h, 6h, 12h), without and with 125mM NaCl. At the start of the process, 0-1 hours in reaction mixture without NaCl, and 0-2 hours in reaction mixture without 125mM NaCl, the ARSF1 sulfatase activity increased. When \ln (residual specific activity) was plotted against incubation time, a linear relationship was observed that showed that ARSF1 sulfatase follows first order deactivation kinetics after 1 h pre-incubation (without NaCl in reaction

mixture) and after 2 h (with 125 mM NaCl in reaction mixture). Based on the slope obtained, it was possible to calculate the first-order rate constant of the thermal denaturation (k_d) for 68°C (Fig 3.7). The half-life ($t_{1/2}$ time it takes for enzyme activity to reduce to half of the activity) was calculated as $\ln 2/k_d$. The $t_{1/2}$ of ARSF1 at 68°C was still 12h and 8h without and with 125mM NaCl, respectively.

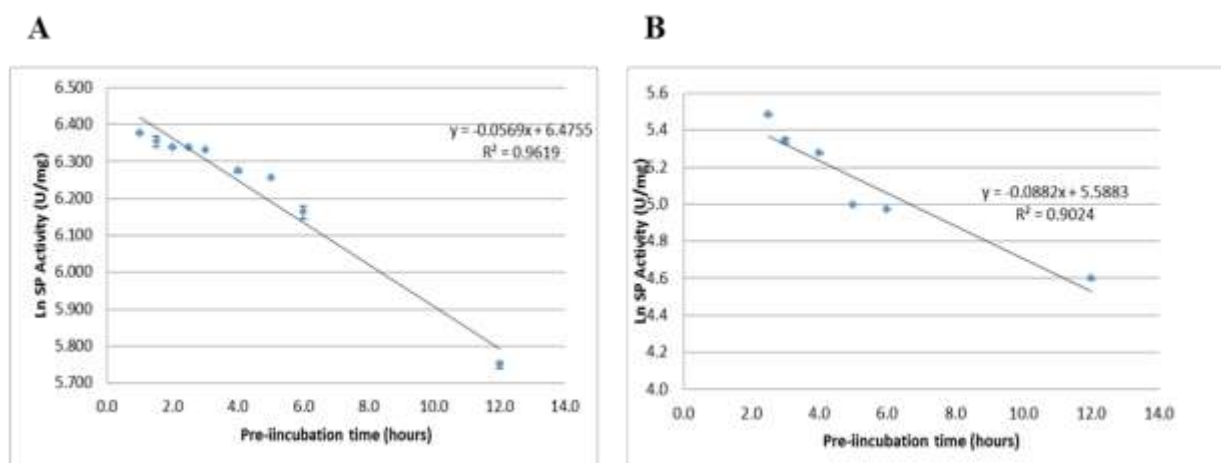


Figure 3.7 Thermal stability of the recombinant ARSF1. A) Reaction mixture without NaCl, B) Reaction mixture with 125mM NaCl. The residual activity of ARSF1 was determined by pre-incubation of ARSF1 in the absence of substrate at 68°C for different times. A semi logarithmic linear plot was obtained for time vs. the Ln of the specific activity (SP Activity, U/mg). The first order rate constant of the thermal denaturation (k_d) was obtain from the slope.

3.4.5 Desulfation kinetics of ARSF1 sulfatase

Michaelis-Menten kinetics were performed for the ARSF1 sulfatase at 68°C in Tris-HCl buffer pH 6.5 and 10mM CaCl_2 with pNCS as substrate in the presence of 125 mM NaCl, and without NaCl (control), and pNCP formation followed by absorption at 515 nm. The results are presented in Table 3.3.

Table 3.3 K_M and V_{\max} of Ps 1595 by Michaelis-Menten kinetics

NaCl(mM)	K_M (mM)	V_{\max} ($\mu\text{M}_{\text{pNCP}}/\text{min}$)
0	0.95 ± 0.15 (100%)	26.6 ± 1.2 (100%)
125	0.88 ± 0.25 (92%)	19.9 ± 1.6 (75%)

3.5. Desulfation of fucoidan by ARSF1 sulfatas

Fucoidan from *S. mcclurei* and *F. evanescens* purified further by ion-exchange chromatography (Pham et al., 2013) (M. Kusaykin et al., 2008) and products of the enzymatic reaction of

fucoidanases FcnA Δ 229, Fda2 and FdlB (Chapter 5), were used as the substrates for ARSF1 sulfatase. The sulfate contents were analyzed by high performance anion exchange chromatography (HPAEC). The results are presented in Fig 3.8 and Table 3.4.

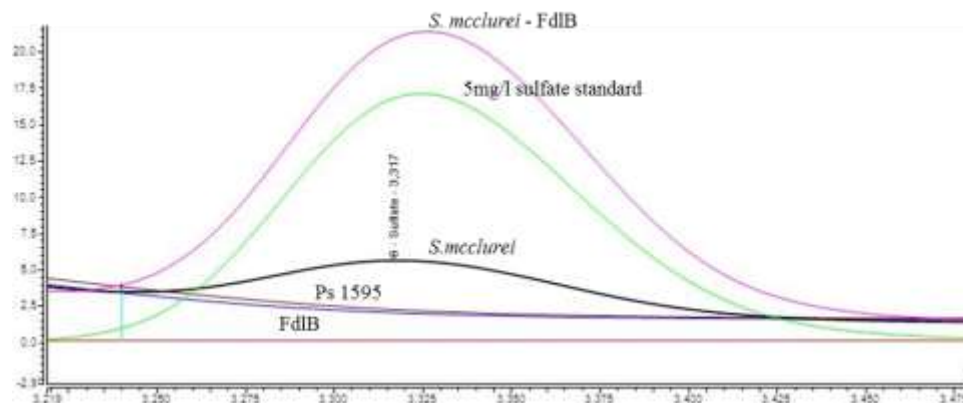


Figure 3.8 Sulfate content of enzymatic reaction of ARSF1 and different fucoidans. Black curve - *S. mcclurei* fucoidan; pink curve products - *S. mcclurei* fucoidan – FdlB; green curve – sulfate standard by high performance anion exchange chromatography (HPAEC).

The results showed that ARSF1 did not work on natural fucoidan from *S. mcclurei* and *F. evanescens*. ARSF1 exhibited the highest activity of 2.95% sulfate released on products of *S. mcclurei* and FdlB (Table 3.4)

Table 3.4 The activity of Ps 1595 on fucoidan substrates

Substrates	SO ₄ released (%)
Fucoidan from <i>S. mcclurei</i>	0.00
<i>S. mcclurei</i> – FcnA Δ 229	0.62
<i>S. mcclurei</i> – Fda2	0.92
<i>S. mcclurei</i> – FdlB	2.95
Fucoidan from <i>F. evanescens</i>	0.00
<i>F. evanescens</i> - FcnA Δ 229	0.24
<i>S. mcclurei</i> – Fda2	0.00
<i>S. mcclurei</i> – FdlB	0.47

3.6. Discussion and conclusions

As we mentioned in the chapter 2, there are seven putative sulfatase from *Pseudoalteromonas* sp. MB47, but one of them are not belonging to the arylsulfatases, contains 5 predicted TMs and no active site conserved domain. Thus, in this study we performed expression of nine putative sulfatases, including six sequences from *Pseudoalteromonas* sp. MB47 (Table 3.1). They belonged to the S1 sulfatase family according to SulfAtlas databases (<http://abims.sb-roscoff.fr/sulfatlas/>)

because the conserved domain CTAGRAALITG was present in all sequences, except for Ps3148 and AER35705 which belong to the amido-hydrolases super family. However, just two sulfatases, including ARSF1 *Pseudoalteromonas* sp. MB 47 and ARS from *Pseudomonas aeruginosa* PAO1, showed activity on pNCS even all of them were expressed well in *E.coli*. This can be explained by the low rate of post-translation maturation of sulfatase in the *E.coli* system. Co-expression of sulfatase with known mature enzymes such as FGE and AnSME might produce active enzymes but the sulfatase maturing system is not yet clearly understood'. Thus, in our study we first focused on studying the active sulfatase ARSF1 and the positive control of sulfatase ARS.

The recombinant arylsulfatase ARSF1 was purified well and had the expected size of 57kDa as determined by SDS-PAGE and western blot. The 3D homology model of ARSF1 showed a dimeric structure and the expected β -sandwich fold (Fig 3.4). In both monomers a calcium ion was bound to the active site. Therefore this model suggests a calcium dependent sulfatase mechanism as in S1 family sulfatases. This finding is also supported by the experimental results of the effects of divalent cations on sulfatase activity. The strong enhancing effect of Ca^{2+} , Mg^{2+} and Mn^{2+} on the ARSF1 enzyme activity observed in this study indicates that divalent cations participated in the catalytic process. Ca^{2+} , Mg^{2+} and Mn^{2+} presumably stabilize negative charges which develop in the sulfate during nucleophilic attack by arylsulfatase of the hydroxyl group of FGly. According to the mechanism of sulfate ester cleavage proposed by Schmidt et al. (Figura et al., 1998), the hydrate form of the arylsulfatase active site FGly attacks the sulfate ester, leading to breakage of the S-O bond and formation of a covalent enzyme-sulfate intermediate.

The optimal conditions for arylsulfatase ARSF1 activity for hydrolysis of sulfate ester bonds in pNCS was pH 6.5 and 68°C. As regards pH optimum, ARSF1 belongs to group of optimal activity at pH values of 6.5–7.1 (Kertesz, 2000). The ARSF1 has an optimal pH similar to that of *S. typhimurium*, although they have different MW and kinetic properties, with a Michaelis constant of 4.1 mM for nitrocatechol sulfate (Henderson and Milazzo, 1979). It is very interesting that ARSF1 exhibited optimal arylsulfatase activity at 68°C and still retained more than 50% activity after 12 hours treatment at optimal temperature while *Pseudoalteromonas* sp. MB47 comes from the sea with water temperatures no higher than 35°C (Vietnam sea). These results are comparable to the previous discovery of a *P. aeruginosa* arylsulfatase which was most active at 57°C (Beil et al., 1995) and of arylsulfatase from a deep-sea bacterium, *Flammeovirga pacifica* (Gao et al., 2015), which was most activity at 40°C. So ARSF1 is not the first thermostable sulfatase obtained from a

bacterium living at much lower temperatures. The finding could be explained by the assumption that this bacterium lived in hot springs a long time ago and somehow this enzyme did not mutate and lose the ability to easily adapt with change of environment. And perhaps, if this enzyme is mutated for no thermal stable, it could lose the sulfatase activity and then it did not happen. This assumption needs further study through discovery of thermal mutants of sulfatase. However, to the best of our knowledge, only two thermal sulfatase sequences have been reported, including an arylsulfatase from *T. maritima* (NP_229503) (Lee et al. 2013) and an arylsulfatase Ary423 from *Flammeovirga pacifica* (AKL72071.1) (Gao et al., 2015). The alignment results from them showed that ARSF1 shared 18.86% amino acid identity with the arylsulfatase from *T. maritima* and 27.84% with Ary423 from the deep-sea bacterium *F. pacifica*.

NaCl concentration also affected ARSF1 sulfatase activity, and presence of NaCl in reaction mixture slowed down sulfatase activity. Without NaCl in reaction mixture, the maximum rate was reached in 30 min, while at 62.5 mM and 125 mM NaCl, the maximum was reached after approximately 50 min or 60 min, respectively. Further studies on the higher NaCl concentrations are necessary to confirm this point. In our study, K_M and V_{max} were also determined by the Michaelis-Menten model. The fitted parameters in the absence of NaCl give a K_M value of 0.95 mM pNCS and a V_{max} of 26.6 μ M pNCS products formed per minute. In the presence of 125 mM NaCl, K_M did not change significantly compared to K_M in the absence of salt. However, a significant effect can be seen on V_{max} where the addition of salt leads to a 25% lower V_{max} compared to the value without NaCl. These results indicate that NaCl does not hinder substrate binding, but rather influences the catalytic step and thus acts like a non-competitive inhibitor, i.e. where NaCl can bind both to the free enzyme but also to the enzyme-substrate complex. With these results, we can use the 0-125 mM NaCl in a reaction mixture for desulfated fucoidan with maybe longer incubated times. ARSF1 sulfatase under optimal condition (pH 6.5, 10 mM $CaCl_2$, 68°C, with and without 125 mM of NaCl) was tested for ability to desulfate natural fucoidan from *S. mcclurei* and *F. evanescens* but activity was not observed. However, ARSF1 exhibited sulfatase activity on the oligosaccharides produced by FcnA Δ 229 and FdlB (Table 3.4). The results suggest that ARSF1 sulfatase does not work on high molecular weight substrates and therefore it cannot help fucoidanase work well. By contrast, fucoidanase catalyzed the hydrolysis of fucoidan to produce oligo-products with lower molecular weight that sulfatase can work on. These results are contrary to our hypothesis that the sulfatase through acting first on fucoidan will create more space for fucoidanase to access fucoidan molecular. For example, a glycosulfatase from *P. vulgata* rapidly

desulfated fucooligosaccharides (Lloyd, 1963). Sulfatase from the marine bacterium *Vibrio* sp. N-5 eliminated sulfate from sulfated fucose and short fucooligosaccharides formed by fucoidanases but its activity towards native fucoidan was very limited (Furukawa et al. 1992a). A partially purified preparation from the bivalve mollusk *P. maximus* effectively desulfated the synthetic substrate p-nitrocatecholsulfate, sulfated at C2 L-fucose, and a natural fucoidan from *A. nodosum* of 13Kda. Sulfatase hydrolyzing p-nitrophenylsulfate rather than natural fucoidan was found in the hepatopancreas of the marine mollusks *Haliotis* sp. and *L.kurila* (Kusaykin et al., 2006).

However, until now there have been two recombinant sulfatase that cleave SO₄ groups of algae polysaccharides are AER35705.1 that active on carrageenan (Genicot et al., 2014) and arylsulfatase AHY96128.1 that active on agar. Both of them come from *Pseudoalteromonas carrageenovora* (Genicot et al., 2014). No sequences of fucoidan sulfatases have been reported previously, thus our ARSF1 is the first one that has action on fucoidan substrate.

There are no commercial sulfated oligofucoidan standards in the present time, so therefore the effort to produce oligofucoidan is considerable. Moreover, we would also like to produce biologically active homogenous fucooligosaccharides. Thus, in the next chapter, we will present the enzymes (including FcnAΔ229 and FdlB were mentioned above) that can modify fucoidan to create the low molecular weight fucooligosaccharides. The oligosaccharides might be further modified with the use of sulfatases to obtain more bioactive fucooligosaccharides.

Chapter 4: Novel enzyme actions for sulfated galactofucan polymerization

Fucoidan from *S. mcclurei* is a special type of sulfated galactofucan of particular interest in Vietnam because *S. mcclurei* is a prevalent type of brown seaweed in Vietnam. The *S. mcclurei* galactofucan has moreover been found to have anti-cancer and anti-virus properties and the structure has been resolved at Nhatrang institute of Technology Research and Application, Vietnam (Pham et al., 2013) (Thanh et al., 2013). This fucoidan was applied in functional foods such as the Fucogastro and Fucoantik products of the Vietnam Fucoidan Company. Hence, to develop this fucoidan to be applied in the medical field, the oligo products should be studied in detail and analyzed for bioactivity. In the present work, the aim of our project is to find the enzymes that are able to degrade fucoidan from *S. mcclurei*.

The objectives of the work are following:

- To identify the fucoidan modifying enzymes in the CAZy database as well as in published papers and patents.
- To express and purify all fucoidan-degrading enzymes.
- To assess the successful recombinantly expressed microbial fucoidan degrading enzymes on fucoidans from *S. mcclurei* and others varying backbone constitution from different brown macroalgae such as *Fucus evanescens*, *Fucus vesiculosus*, *Turbinaria ornata*, *Saccharina cichorioides* and *Undaria pinnatifida*.

The study was investigated based on hypothesis (3) stated on page 2 of this thesis:

Fucoidan from *S. mcclurei* has both $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ linked fucosyl residues and galactosyl- $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages in the backbone. We hypothesize that fucoidanases with either endo $\alpha(1\rightarrow3)$ or $\alpha(1\rightarrow4)$ activity might cleave the linkages in the backbone of this fucoidan. In addition, the structure of *S. mcclurei* has not yet been discovered completely, so using specific enzymes might give us more information about fucoidan structure. Hence, fucoglucoromanan lyase was also used in our study.

This study is related to the paper: Novel enzyme actions for sulfated galactofucan depolymerisation and a new engineering strategy for molecular stabilization of fucoidan degrading enzymes.

4.1. Gene identification

We took five enzymes, FcnA, Fda1, Fda2, FdlA and FdlB, from GenBank (Table 4.1). Among them, the recombinant FcnA was previously reported not to have activity so the C-terminal

truncated version of FcnA called FcnA2 was created by Colin et al in 2006 (Colin et al., 2006). Hence, we used FcnA2 in our study.

The optimized codon genes were optimized for the expression in *E. coli*. They were synthesized by GenScript (Piscataway, NJ, USA) and inserted in the pET-45b (+) vector between the KpnI and PacI (FcnA2) and pET-19b (+) vector between NcoI and XhoI limited site.

Table 4. 1. Fucoidan-degrading enzymes, features, molecular weight, and expression strains used.

Enzyme name/ GenBank no.	Organisms	Length (aa) ^b	Expected mol. weight (kDa)	<i>E.coli</i> expression strain
FcnA CAI47003.1	<i>Mariniflexile</i> <i>fucanivorans</i> SW5	1007	nd	Nd
FcnA2	<i>Mariniflexile</i> <i>fucanivorans</i> SW5	799	88	BL21 (DE3) pGro7 ^c
Fda1 AAO00508.1	<i>Alteromonas</i> sp. SN-1009	804	87	BL21 (DE3) pGro7 ^c
Fda2 AAO00509.1	<i>Alteromonas</i> sp. SN-1009	868	94	BL21 (DE3) pGro7 ^c
FdlA AAO00510.1	<i>Flavobacterium</i> sp. SA-0082	684	75	C41 (DE3)
FdlB AAO00511.1	<i>Flavobacterium</i> sp. SA-0082	692	76	C41 (DE3)

nd: not determined in this study.

^a Wild type signal peptide had been removed for codon-optimized synthesized construct.

^b Including the his-tags

^c groES-groEL chaperone expressed from the pGro7 plasmid

4.2. Recombinant enzyme expression

The expression of FcnA2, Fda2, FdlA and FdlB was successful and the purified enzymes were evaluated by SDS-PAGE and western blot (Fig 4.1A, B). The expression of Fda1 was also high but the protein was retained in the cell debris after sonication (Fig 4.2). The FdlA and FdlB showed one band on both the SDS-PAGE gel and the western blot, as expected of size 75 and 76 kDa, respectively. Fda2 expressed with a high level of degradation and migrated more slowly in the SDS-

PAGE gel than expected (94kDa). FcnA2 and Fda2 showed more than one visible band in both the SDS-PAGE gel and in the western blot (Fig 4.1A, B).

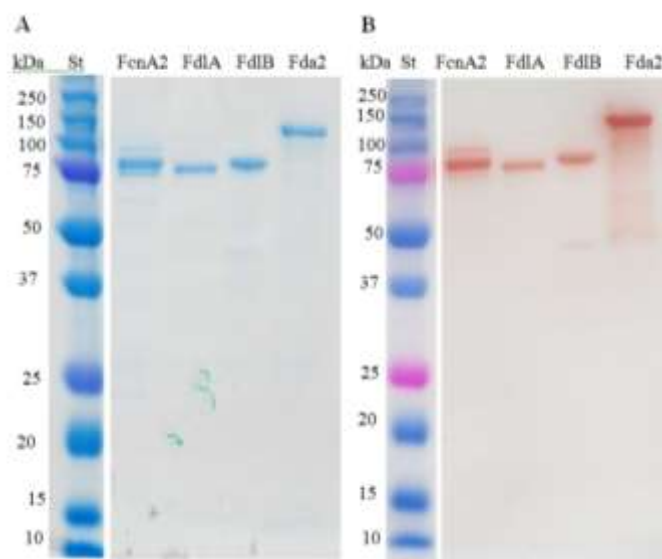


Figure 4.1 Purified recombinantly expressed fucoidan-modifying enzymes. A) SDS-PAGE and B) Western blot of purified FcnA2, FdlA, FdlB and Fda2. (St) is the protein plus molecular weight marker. The expected molecular weights of the recombinant enzymes FcnA2, FdlA, FdlB and Fda2 were 87, 75, 76, and 94 kDa, respectively. The multiple bands seen for FcnA2 and Fda2, notably in the western blot, indicate partial degradation of the proteins.

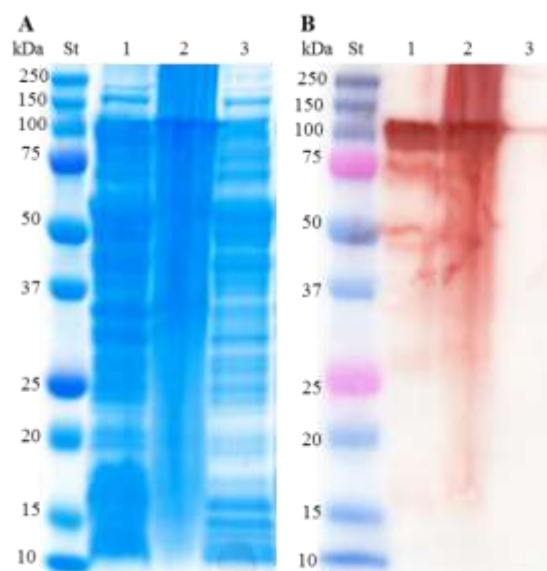
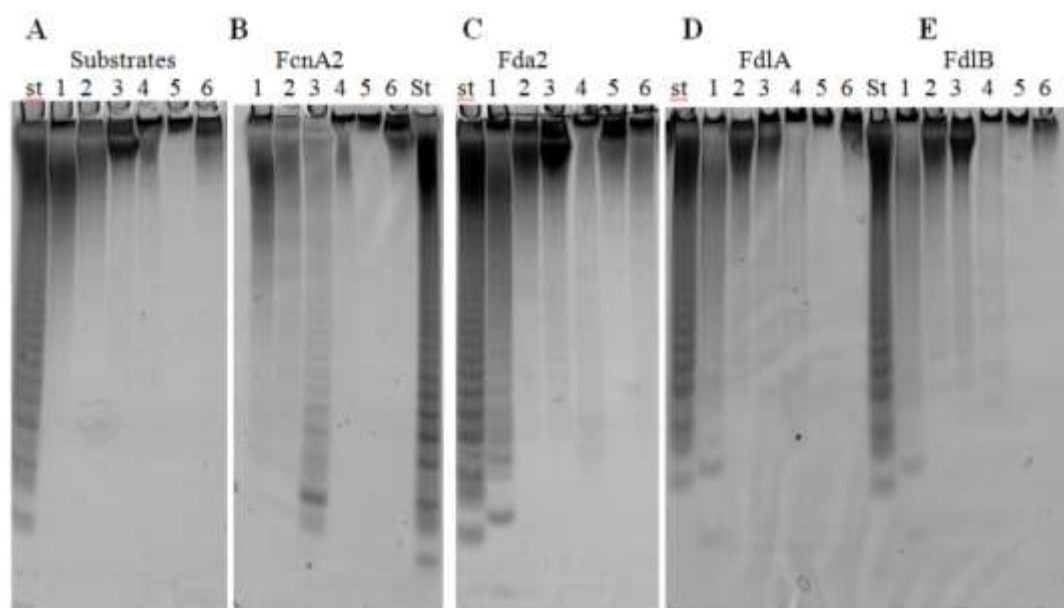


Figure 4.2 Recombinant expression of Fda1 in *E. coli*. A) SDS-PAGE and B) western blot of 1) Auto induced cells; 2) the cell debris (after sonication and protein extraction); 3) crude extract after sonication and centrifugation. (St) is the protein plus molecular weight marker.

4.2. Substrate specificity of the recombinant fucoidan-degrading enzymes

Six different fucoidan samples from *Sargassum mcclurei* (1), *Fucus vesiculosus* (2), *Fucus evanescens* (3), *Turbinaria ornata* (4), *Saccharina cichorioides* (5), and *Undaria pinnatifida* (6) were treated with the individual enzymes FcnA2, Fda2, FdlA and FdlB under condition of 20mM Tris-HCl buffer pH 7.4, 250mM NaCl and 10mM CaCl₂, 1% fucoidan (weight/volume) and incubated at 35°C for 24 hours. The fucoidan-degrading enzyme profiles were obtained using Carbohydrate-Polyacrylamide Gel Electrophoresis (C-PAGE) (Fig 4.3).



Enzymes	Substrates					
	<i>Sargassum mcclurei</i>	<i>Fucus vesiculosus</i>	<i>Fucus evanescens</i>	<i>Turbinaria ornata</i>	<i>Saccharina cichorioides</i>	<i>Undaria pinnatifida</i>
FcnA2	+	++	+++	-	-	-
Fda2	+++	+	+	+	(+)	(+)
FdlA	+++	+	+	+	-	-
FdlB	+++	+	+	+	-	-

Figure 4.3 C-PAGE product profiles of fucoidan degradation using purified enzymes. A) Substrate control; B, C, D and E) Enzymatic reaction of FcnA2, Fda2, FdlA and FdlB on different fucoidans, respectively: 1) *Sargassum mcclurei*; 2) *Fucus vesiculosus*; 3) *Fucus evanescens*; 4) *Turbinaria ornata*; 5) *Saccharina cichorioides*; 6) *Undaria pinnatifida*. The lowest band of the standard (St) resulting from FFA2 treatment of fucoidan from *F. evanescens* corresponds to a tetra-saccharide of (1→4) and (1→3)-linked α-L-fucosyls with each fucosyl residue sulfated at C2 (Silchenko et al. 2017). The extent of degradation is indicated as: +++ highest, ++ medium, + lowest, and (+) is positive activity resulting in a high molecular smear; (-) is no activity. The reaction time was 24 hours.

The data presented in Fig 4. 3 indicate more effective degradation of the fucoidan substrates from *S. mcclurei* (1), *F. vesiculosus* (2) and *F. evanescens* (3) than of substrates with predominantly $\alpha(1\rightarrow3)$ glycoside bonds in their backbone structures, which originate from *T. ornate* (4), *S. cichorioides* (5) and *U. pinnatifida* (6).

4.3. Further assessment of *Sargassum mcclurei* fucoidan degradation

C-PAGE and SEC of oligosaccharides released by FcnA2, Fda2, FdlB and FdlA after extended reaction for 48 hours showed that each enzyme catalyzed effective degradation of *S. mcclurei* fucoidan (Fig 4.4).

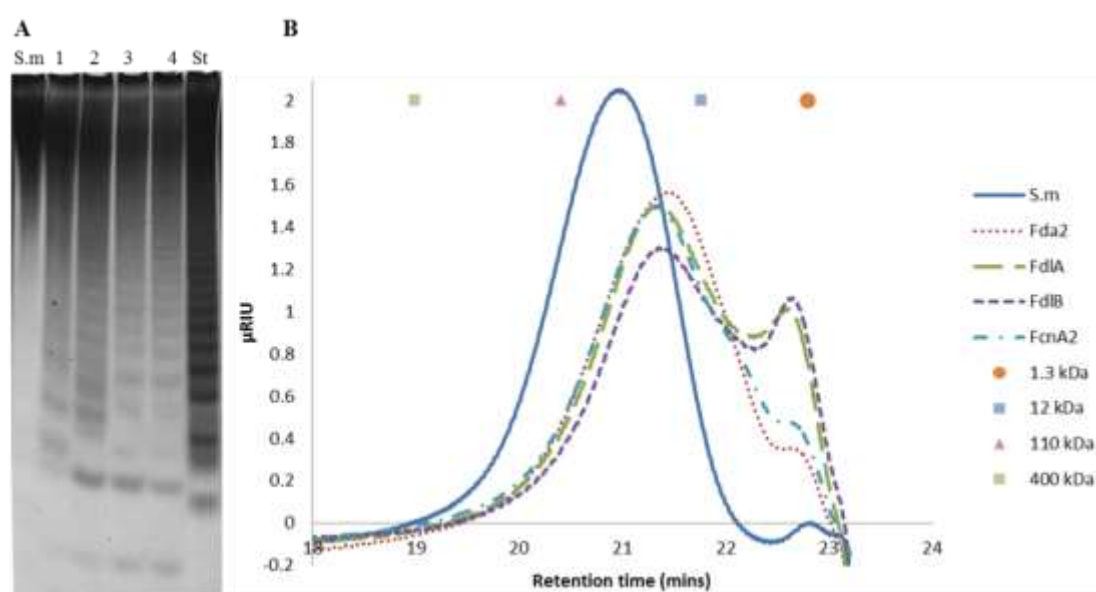


Figure 4.4 *Sargassum mcclurei* fucoidan (S.m) degraded by fucoidanases. A) C-PAGE and B) Size exclusion chromatography (SEC) of 1) FcnA2, 2) Fda2, 3) FdlA, 4) FdlB on *S. mcclurei* fucoidan and molecular weight standards. Reaction time was 48 hours. (St) in C-PAGE is a hydrolysis standard from FFA2 treatment of fucoidan from *F. evanescens* (Silchenko et al. 2017).

4.5. Discussion and conclusion

In this study we successfully expressed FcnA2, Fda1, Fda2, FdlA and FdlB in the *E.coli* system. The high purity level of FdlA and FdlB with the expected sizes of 75 and 76 kDa, respectively, (Fig 4.1 A, B) render them suitable for modifying fucoidan. For FcnA2, the multiple bands of purified enzyme were observed in SDS-PAGE. This result agrees with previously published data for recombinantly expressed FcnA2 by Colin et al. in 2006. Authors reported that the multiple bands of FcnA2 happened because of the “co-elution” with other proteins, which could not be separated by

anion exchange or SEC (Colin et al., 2006). However, in our study, the multiple bands of FcnA2 observed also in the western blot assay indicated spontaneous degradation rather than impurities from other proteins. Similar FcnA2, for Fda2, the multiple bands observed in the western blot indicated spontaneous degradation and this process happened during expression in *E. coli* cells, during the purification process, and during storage. And for Fda1, the appearance of multiple bands in the cell debris western blot indicates that the tagged Fda1 degraded inside the cells (Fig 4.2); the intensity of the bands suggests that the recombinant enzyme was expressed but that the expressed enzyme was not soluble and hence was probably retained in inclusion bodies. Therefore stabilization of FcnA2, Fda1 and Fda2 to avoid the degradation and make them more soluble will be necessary for studying their activity; this problem is addressed in the next chapter. Although degradation of FcnA2, Fda2, occurred, they were still used to test fucoidan activity on various structural fucoidans, including fucoidan from *S. mcclurei*.

In this study, we used the C-PAGE method to test for recombinant fucoidan-modifying enzyme activity because the reducing sugar method did not succeed and because of limited substrate. The difficulties of the C-PAGE method were that we did not have any commercial oligo-sulfated fucose standards and therefore we could not accurately quantify the products. The standard we used in this study was a mixture of products of enzymatic reaction of FFA2 and *F. evanescens* that was kindly supplied by Silchenko et al. (Silchenko et al. 2017). We used this standard as the positive control in the C-PAGE method as well as for comparison of the C-PAGE products with other enzymatically obtained products. The smallest band of this standard was characterized as a tetra-oligosaccharide sulfated at C2 of all fucose residues. Thus, based on the information of the standard, the presence of enzymatic product bands in the same gel allows semi-quantification of monosaccharides and oligosaccharides. The results of the enzyme activities investigated are presented in Fig 4.3.

It is interesting that all enzymes showed activity on fucoidan from *S. mcclurei* (Fig 4.3). These results were also supported by the Size Exclusion Chromatography (SEC) method of enzymatic reaction after 48 hours reaction (Fig 4.4). The C-PAGE profiles were more visible after 48 hours (Fig 4.4) than after 24 hours (Fig 4.3) reaction time. While pre-incubation enzymes even at 4°C, 48 hours led to loss enzyme activity. These results indicated that the enzymes were more stable when combined to substrates.

By C-PAGE, the smallest oligosaccharide of enzymatic reaction ran further than the lowest one of the standard (Fig 4.4 A). This result suggests that the released smallest oligosaccharide products

were either smaller or more charged, i.e. more sulfated, than the tetra-saccharide in the standard. The SEC profiles of FcnA2 and Fda2 were similar, but the product profile differed from those of FdlA and FdlB in having a smaller peak at ~22.5 min. (corresponding to a molecular weight SEC standard of ~1.3 kDa). In contrast, FdlA and FdlB both had a peak in the SEC at around 22.5 min, indicating that they acted slower if at all on certain fucoidan fragments ≤ 1.3 kDa. Taken together with the C-PAGE results (Fig 4.3A), these data suggest that FdlA and FdlB exerted similar substrate attack preferences and left behind some oligomers of around 1.3 kDa, whereas FcnA2 and Fda2 appeared to degrade the lower molecular weight oligomers to a more significant extent. The action of these enzymes on *S. mcclurei* fucoidan is a new finding that shows promise for employing fucoidan-degrading enzymes for controlled degradation of the complex galacto-fucan fucoidan from *S. mcclurei*.

The specific substrate of these enzymes was assessed by treating different structural fucoidans with them. The recombinantly expressed FcnA2 enzyme showed activity on both substrates from *F. evanescens* and *F. vesiculosus* that have similar alternating $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ glycoside bonds in the backbone (Fig. 4.3A). The apparent lack of action of FcnA2 on the fucoidan from *T. ornata*, *S. cichorioides* and *U. pinnatifida*, which all have $\alpha(1\rightarrow3)$ linkages, suggests that the FcnA2 does not catalyse cleavage of $\alpha(1\rightarrow3)$ bonds between fucose residues whereas the activity on the other three substrates supports the interpretation that the enzyme attacks $\alpha(1\rightarrow4)$ bonds, as previously shown (Colin et al., 2006). On the other hand, the more C-PAGE profiles from *F. evanescens* than *F. vesiculosus* indicates a difference in fucoidan structure, even if they both contain $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ linkages. FcnA2 was also reported to be able to degrade fucoidan from *Pelvetia canaliculata*, which has the same backbone structure as the two *Fucus* fucoidans (Colin et al., 2006). Fucose residues of fucoidan from *P. canaliculata* and *F. evanescens* were sulfated at C2/C3 (C2/C4) and C2, while *F. vesiculosus* was more sulfated at C2 and C4, and even sulfation at C3 of the fucosyl residue. Thus the lack of C2/C4 and C2/C3 disulfation in *F. vesiculosus* fucoidan might be the reason for weaker degradation of fucoidan from *F. vesiculosus* than from *F. evanescens* (Fig. 4.3A). The differences in degradation of fucoidan from *F. evanescens* and *F. vesiculosus* therefore indicate that differences in the sulfation pattern or in other types of substitutions on the substrate backbones may influence the action of FcnA2 on fucoidans derived from these two *Fucus* spp. The smallest oligomers released from *F. evanescens* by FcnA2 and standard (st) were different (Fig. 4.3). The difference in the released oligomers indicated that the sulfation preferences of FFA2 and

FcnA2 may differ. The partial degradation of *S. mcclurei* fucoidan can be explained by attack of FcnA2 at the $\alpha(1\rightarrow4)$ linked (sulfated) L-fucose residues, which is the enzyme action previously found for this enzyme (Colin et al., 2006).

C-PAGE profiles of the treatment of different substrates with Fda2 were shown in Fig 4.3C. The results also showed that this enzyme exerted a weak and almost identical degree of degradation of the fucoidans from *F. vesiculosus* and *F. evanescens* which contain alternating $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ glycosidic bonds between the α -L fucosyl residues in their backbone structures. Fda2 had low activity on the fucoidans from *T. ornata*, *S. cichorioides* and *U. pinnatifida* which are rich in $\alpha(1\rightarrow3)$ fucosyl linkages (Fig 4.3C). Hence the activity of the enzyme on *S. mcclurei*, *F. evanescens* and *F. vesiculosus* together with the weak activity on substrates rich in $\alpha(1\rightarrow3)$ fucosyl linkages corroborated previous claims of the action of Fda2 on $\alpha(1\rightarrow3)$ bonded L-fucosyls in fucoidan (Takayama et al., 2002).

In this study, FdlA and FdlB were originally derived from *Fucobacter marina* SA0082 and both exerted activity on fucoidans from *S. mcclurei* and *F. evanescens* but only very weak activity on fucoidan from *F. vesiculosus* and *T. ornata*. FdlA and FdlB were claimed to be fucoglucoromanan lyases on the basis of increased UV232 nm absorbance due to the double bond and NMR analysis of oligoproducts (Takayama et al., 2002). However, in our study, no lyase action was detected since there was no increasing UV232 nm absorbance of the enzymatic reaction even though the investigated fucoidans had both mannan and uronic acids in their composition. The explanation for this may be that fucoidan preparations from *S. mcclurei*, *T. ornata*, *F. evanescens* and *F. vesiculosus* contain very low amounts of uronic acid and sometimes traces of mannose (Pham et al., 2013), (Ermakova et al. 2015b), (Zvyagintseva et al., 2003), (Nishino et al., 1994) and until now no data show that D-mannosyl and D-glucuronate are present in the backbone of these fucoidans. FdlA and FdlB might have another type of action that was reported to cleave O-glycosidic bonds between D-mannose (D-man) and D-glucuronic acid (D-GlcA) residues in the mamnuronane backbone of the fucoidan (Takayama et al., 2002). There was no increasing UV232 nm absorbance of the enzymatic reactions might also be explained that our fucoidan substrates had a little brownness. The colour might interfere with the assay then the double bond assay did not work in our experiments.

On another hand, the lack of activity on fucoidan from *S. cichorioides* and from *U. pinnatifida* fucoidan indicated that FdlA and FdlB do not cleave $\alpha(1\rightarrow3)$ bonds in fucoidan. Hence, FdlA and FdlB most likely cleave $\alpha(1\rightarrow4)$ fucosyl bonds in the backbone of these fucoidans. The similar

weak extent of degradation of the *F. vesiculosus* and *F. evanescens* fucoidan by both enzymes, to produce almost similar oligomer profiles in C-PAGE, suggests preference for rare or complex (e.g. C2 and C4) fucosyl-sulfation in the *Fucus* fucoidan substrates – maybe of a type which occurs more abundantly in the *S. mcclurei* fucoidan, and most likely attacks only $\alpha(1\rightarrow4)$ fucosyl-bonds. The FdlB appeared to exert a more profound action than the FdlA on the *F. evanescens* substrate.

The action of the two enzymes on *S. mcclurei* galacto-fucan produced a band which moved further in the gel than the sulfated tetra-saccharide of the control, which suggests that both FdlA and FdlB are able to catalyse disintegration of sulfated fucoidan oligomers. Due to the high degree of depolymerisation down to oligosaccharides of less than DP4, due to the high abundance of galactosyl residues in *S. mcclurei* fucoidan, we cannot rule out the possibility that FdlA and FdlB may cleave galactosyl- $\alpha(1\rightarrow4)$ bonds (Fig. 4.3 D, E), although this needs further analysis.

In conclusion we successfully expressed and purified FdlA and FdlB. The stabilization of FcnA2, Fda1 and Fda2 to obtain higher activity is presented in the next section.

Chapter 5. Stabilization of fucoidan degrading enzymes by new engineering strategy for molecular

We discovered that FcnA2 and Fda1, Fda2 were degraded during the expression and purification process leading to loss of activity. Thus stabilization of these enzymes by an engineering strategy was our main aim of this study.

The objectives of the work were the following:

- To analyse the sequences of FcnA2, Fda1 and Fda2 by bioinformatics tools.
- To analyse the protein based on western blot analysis results.
- To make new constructs of those enzymes.
- To express and purify the modified proteins.
- To evaluate the activity of the new enzymes on fucoidan substrates.

The study was based on the following hypotheses

Fucoidanase enzymes are degraded from the C-terminal end, therefore removing the C-terminal mutants to different degrees might make the enzymes more stable.

This study related to the second part of the paper: Novel enzyme actions for sulfated galactofucan depolymerisation and a new engineering strategy for molecular stabilization of fucoidan degrading enzymes.

5.1. New construct of FcnA and the activities

A new construct of FcnA2 was made by removing an additional 80 amino acids from the C-terminal end. This truncated enzyme was thus 229 amino acids shorter than the original FcnA enzyme and was called FcnA Δ 229. FcnA Δ 229 was expressed well and purified to high degree as illustrated by SDS-PAGE and western blot analysis, and gave the expected band size of 80 kDa with no apparent protein degradation (Fig 5.1 A, B). Hence, FcnA Δ 229 recombinant enzyme and FcnA2 were used to test for activity on *S. mcclurei*, *F. evanescens* and *F. vesiculosus* substrates (Fig 5.1C).

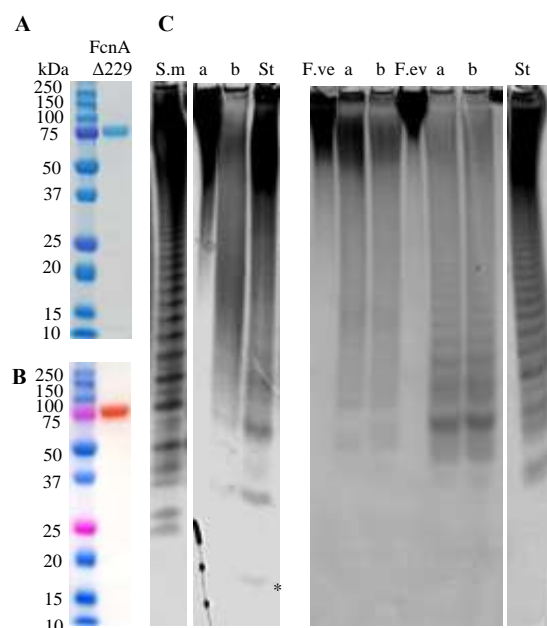


Figure 5.1 Purification and enzyme activity of FcnA Δ 229. A) SDS-PAGE indicating the expected molecular weight of 80 kDa and very pure protein, B) Western blot of purified FcnA Δ 229. (St) is the protein plus molecular weight marker, C) Enzyme activity by C-PAGE of a) FcnA2 and b) FcnA Δ 229 on fucoidans from *S. mcclurei*, *F. vesiculosus* and *F. evanescens*. FcnA2 and FcnA Δ 229 have similar profiles on *F. vesiculosus* and *F. evanescens* fucoidans. The reaction time was 24 hours. *FcnA Δ 229 released an oligosaccharide of lower molecular weight than FcnA2.

5.2. Stabilization through C-terminal truncation of Fda1 and Fda2

The sequence of Fda1 and Fda2 were analyzed and it was found that both enzymes contained two predicted Laminin G domains (IPR001791) (LamG domains) towards the C-terminal end (Fig 5.2).

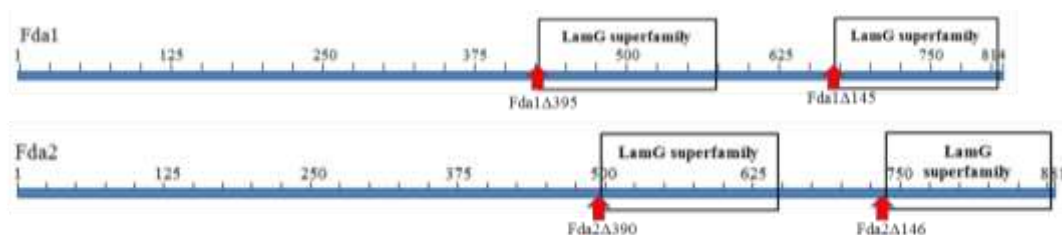


Figure 5.2 Predicted protein domain structures of Fda1 and Fda2. Domains were predicted using Blast, and both proteins contain two predicted LamG (Laminin G) superfamily domains. In Fda1 the domains are from 429 to 574 and 670 to 809. In Fda2 the domains are from 496 to 641 and 737 to 876. Arrows indicate the deletion points. Deletion mutants were named according to deletion from the C-terminal end, i.e. Fda1 Δ 145, Fda1 Δ 395, Fda2 Δ 146 and Fda2 Δ 390.

Moreover, based on western blot analysis of Fda2 (Fig 4.1B – Chapter 4), enzyme destabilization occurred via degradation from the C-terminal as with FcnA2. Therefore a strategy to stabilize the enzymes was carried out involving deletion of the two predicted LamG domains in Fda1 and in Fda2. Hence, new constructs of Fda1, called Fda1 Δ 145 (one LamG domain deleted) and Fda1 Δ 395 (Both LamG domains deleted), were prepared, each with an additional his-tag. The additional his-tag was included as a new strategy in the construct to ensure better binding to the Ni²⁺ Sepharose column.

Fda2 was highly unstable and lost substantial amounts of protein during purification, presumably due to lack of binding to the column. Therefore a construct was made with an additional C-terminal his-tag in order both to stabilize the enzyme and ensure better binding to the column during purification. This new construct was called Fda2-His. In addition, as for Fda1, new constructs devoid of either one or both of the two predicted LamG domains of Fda2 were also made. These Fda2 C-terminal deletion mutants were called Fda2 Δ 146 and Fda2 Δ 390 (Fig 5.2).

All truncated enzymes of Fda1 and Fda2 were well expressed (Fig 5.3) and their purification and activities were presented in Fig 5.4. Some protein degradation was still evident, but notably the double LamG deletion constructs, Fda1 Δ 395 and Fda2 Δ 390, appeared more stable than the less truncated enzymes (Fig 5.4A, B). All the truncated enzymes exerted activity on *S. mcclurei* fucoidan (Fig 5.4C).

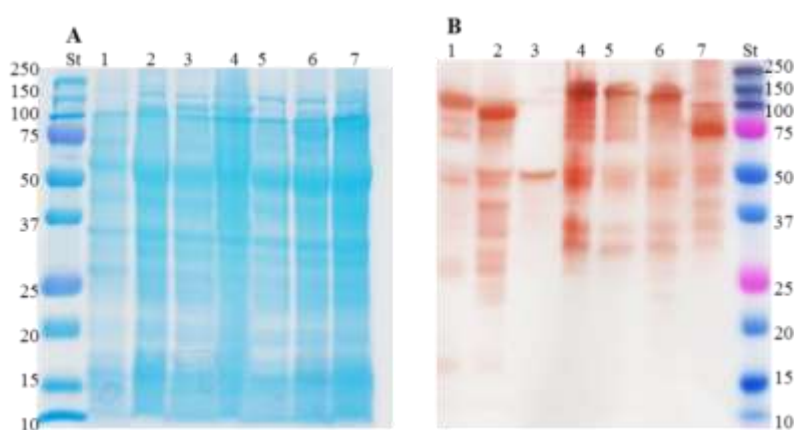


Figure 5.3 The expression of truncated Fda1 and Fda2 in E.coli. A) SDS-PAGE and B) western blot of induced cells of 1) Fda1; 2) Fda1 Δ 145; 3) Fda1 Δ 395; 4) Fda2-His, 5) Fda2; 6) Fda2 Δ 146; 7) Fda2 Δ 390. St is the protein plus molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA). Degradation is already evident before sonication

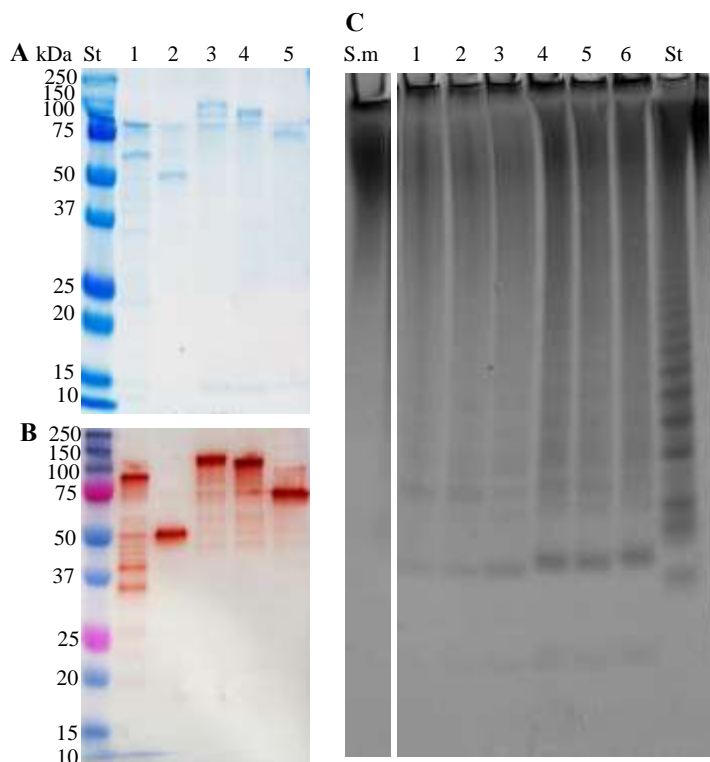


Figure 5.4 Purification of deletion mutants of Fda1 and Fda2 and their activity. A) SDS-PAGE and B) western blot of purified 1) Fda1 Δ 145, 2) Fda1 Δ 395, 3) Fda2-C-His, 4) Fda2 Δ 146, 5) Fda2 Δ 390. (St) is the protein plus molecular weight marker. The expected sizes of the proteins were 90, 50, 125, 110, 70 kDa, respectively. C) Enzymatic *S. mcclurei* fucoidan (S.m) degradation by C-PAGE: 1) Fda1 Δ 145, 2) Fda1 Δ 395; 3) Fda2-His; 4) Fda2 Δ 146; 5) Fda2 Δ 390; 6) Fda2 and the standard (St) resulting from FFA2 treatment of fucoidan from *F. evanescens*. The reaction time was 48 hours.

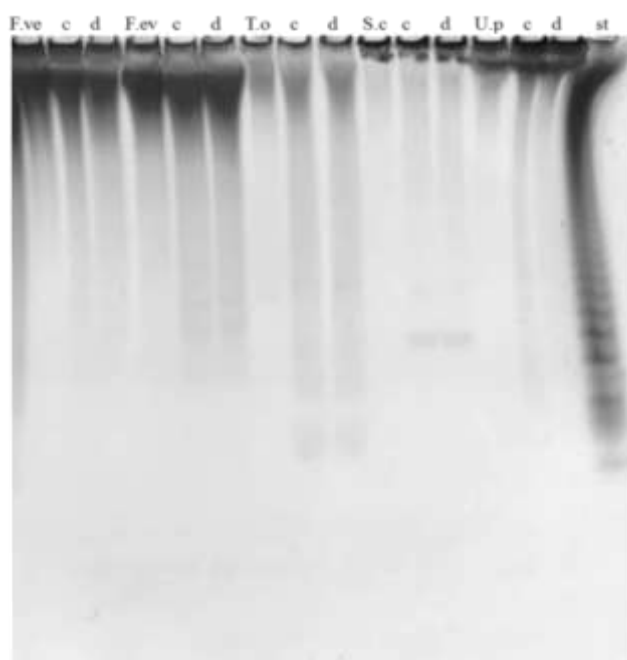


Figure 5.5 Enzyme activity of truncated Fda1 mutants by C-PAGE. Enzyme activity of c) Fda1 Δ 145 and d) Fda1 Δ 395 on fucoidans from *F. vesiculosus* (F.ve), *F. evanescens* (F.ev), *T. ornate* (T.o), *S. cichorioides* (S.c) and *U. pinnatifida* (U.p), standard (st). Both enzymes show activity on all the tested substrates to a comparable degree.

C-terminally truncated Fda1 showed activity on fucoidans from *F. vesiculosus*, *F. evanescens*, *T. ornate*, *S. cichorioides* and *U. pinnatifida* (Fig 5.5)

5.3. Discussion and conclusions

The new construct, FcnA Δ 229, of FcnA2 could be expressed very well and purified to high degree to a size of 80 kDa with no apparent protein degradation (Fig 5.1 A, B). The truncated enzyme had the same activity on fucoidan of *F. evanescens* and *F. vesiculosus*. However, when degrading fucoidan from *S. mcclurei*, a smaller oligosaccharide was released by FcnA Δ 229 compare to FcnA2 after 24h reaction time (Fig 5.1C). Thus this result indicated that the change in stability conferred by deletion of the 80 amino acids in FcnA2 apparently enhanced substrate degradation, but the truncation did not confer any other apparent changes to the *S. mcclurei* degradation profile. Using Blast in NCBI, FcnA2 was shown to contain the T9SS C-terminal domain. T9SS C-terminal domain was also found in all other fucoidanase sequences from the phylum Bacteroidetes. Proteins secreted by T9SS have amino-terminal signal peptides for export across the cytoplasmic membrane by the Sec system and carboxy-terminal domains (CTDs) that target these proteins for secretion across the outer membrane by the T9SS (Kulkarni et al., 2017). Thus the T9SS C-terminal domain probably do not contribute to the catalyzing function of the fucoidanases, but this domain might help bacteria orient the food sources and ensure that the products of fucoidanase are released in their immediate surroundings from which the bacteria can easily be absorb the food. The presence of the T9SS domain in the recombinant enzyme might make fucoidanase more bulky, and the active

site might be hidden and might not be accessed by the fucoidan, thus leading to low activity. By removing this domain, the enzyme would show higher activity as was demonstrated in our study. Therefore FcnA Δ 229 with its high degree of expression, purification and activity could be used to crystalize the enzyme and to understand in more detail the structure of this enzyme.

The truncated Fda1 and Fda2 were successfully expressed. However, degradation of the truncated enzymes Fda1 Δ 145, Fda2-C-His, Fda2 Δ 146 and Fda2 Δ 390 was still evident and already had occurred inside the *E. coli* cells before sonication (Fig 5.3). Degradation of Fda1 and Fda2 might be the reason why Fda1 and Fda2 recombinant enzymes have not previously been biochemically characterized. Degradation might also be the reason why the substrate specificity of these enzymes has not been found before now.

The Fda1 Δ 395 that was obtained by LamG double domain deletion showed only one band on SDS-PAGE and western blot, which indicated that this enzyme was stable and still retained activity on fucoidan from *S. mcclurei* (Fig 5.4). On the other hand, both truncated enzymes of Fda1 showed activity on all the tested substrates (*F. vesiculosus*, *F. evanescens*, *T. ornate*, *S. cichorioides* and *U. pinnatifida*) to a comparable degree (Fig 5.5). Hence the data obtained support the interpretation that Fda1 and their truncated enzymes, like Fda2, catalyze cleavage of $\alpha(1\rightarrow3)$ fucosyl bonds in sulfated fucoidan backbones.

For both Fda1 and Fda2, LamG domains seem not to contribute to the catalyzed function of fucoidanase. These Laminin G domains have been found to see vary in their functions, such as cell adhesion, signaling, migration, assembly and differentiation, and a variety of binding functions have been ascribed to different LamG modules (Sasaki et al.2004).

In conclusion, stabilization of the enzymes was successfully achieved by double his-tagging and notably by targeted truncation of the C-terminal ends in FcnA2, Fda1 and Fda2. Interestingly, for FcnA2, stabilization by C-terminal truncation produced an FcnA2 variant, called FcnA Δ 229, which appeared able to foster more effective degradation of *S. mcclurei* fucoidan than the parent enzyme. For Fda1, deletion of 2 lamG domains (47%) produced a variant, Fda1 Δ 395, which was more stable and active. For Fda2 it is necessary to continue studying other functions of this protein to make it more stable and more active.

Chapter 6. Conclusions and perspectives

Fucoidans have attracted a lot of attention in the scientific community and in industry because of the diversity of their biological activities. However, fucoidans have so far been applied only as a functional food, or used in agriculture and in cosmetics. For application in the medical field, more study is needed of the detailed structure of fucoidan along with its precise biological activity. One of the methods for determining the fine structures of fucoidan and for producing oligo products without changing their activity is enzymatically catalyzed degradation. This PhD study highlighted a few of the crucially important factors in using enzymes to modify fucoidan. Important steps were taken towards elucidating fucoidanase-coding genes, such as domain analysis together with protein analysis by western blot. We then obtained good expression of fucoidanase genes in heterologous host organisms. Experiments to over-produce, purify and stabilize the fucoidanases were performed. We also, for the first time, found fucoidan sulfatase coding genes and primary properties. Thus the findings of this current work have potential for studying the biological activity of produced and modified fuco-oligosaccharides to make them suitable for use in industrial medicines.

Nevertheless, further biochemical characterization by NMR analysis of the enzymatic products of the fucoidan sulfatase and fucoidanases is still required using other fucoidan substrates. Likewise, studies are needed where fucoidanase and sulfatase are combined to determine the fine fucoidan structures as well as to create oligosaccharides with and without sulfate groups. Then it would also be interesting to compare the bioactivity of these oligosaccharides to enhance knowledge of the sulfate group role in fucoidan molecules. It is also important to obtain more knowledge about the sulfatase and fucoidanase structure e.g. a crystal structures.

This study also isolated from the gut of sea cucumber new and potentially interesting marine bacteria that produce fucoidanase and sulfatase. This collection can be used for further study to find new fucoidanases and sulfatases. *Cobetia* sp. MB87 which showed higher fucoidanase activity on the *S. mcclurei* is especially very interesting with regard to finding new kinds of fucoidanases (maybe those that attack fucose-galactose linkages). Although we obtained only low activity of the *Cobetia* sp. MB87 fucoidanase, it might be possible to alter performance through the use of an engineering strategy with the other fucoidanases once the sequence of the fucoidanase is obtained.

Other strains that were isolated from sea cucumber gut also showed potential enzymatic activity on fucoidan, indicating that they are likely to produce fucoidanases. These might also be unstable in

experimental set-up and this might be a reason for further studies using the DNA library for screening for activity and eventually obtaining the gene sequence.

In conclusion, the application of fucoidan – degrading enzymes with various specificities allows elucidation of the structure of fucoidans and may help to provide further insight into the biological activity of fucoidans isolated from various sources. In the near future, we believe fucoidanases can be obtained on an industrial scale in order to produce fuco-oligosaccharides for new medicines.

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Chapter 7. Manuscripts

I. Isolation and screening of aerobic marine bacteria from sea cucumber to identify novel fucoidan modifying enzymes

Hang Thi Thuy Cao, Maria Dalgaard Mikkelsen, Mateusz Jakub Lezyk, Nanna Rhein-Knudsen, Bui Minh Ly, Van Thi Thanh Tran, Thuan Thi Nguyen, Thinh Duc Pham, and Anne S. Meyer.

(To be submitted to Enzyme and Microbial Technology)

II. A thermostable fucoidan active sulfatase (co-first author)

Maria Dalgaard Mikkelsen, Hang Thi Thuy Cao, Nanna Rhein-Knudsen, Jesper Holck, Mateusz Jakub Lezyk, Jan Muschiol, Van Thi Thanh Tran, and Anne S. Meyer

(To be submitted to Journal of Biological Chemistry)

III. Novel enzyme actions for sulfated galactofucan depolymerisation and a new engineering strategy for molecular stabilization of fucoidan degrading enzymes

Hang Thi Thuy Cao, Maria Dalgaard Mikkelse, Mateusz Łęzyk, Ly Minh Bui, Van Thi Thanh Tran, Artem S. Silchenko, Mikhail I. Kusaykin, Thinh Duc Pham, Bang Hai Truong, Jesper Holck, and Anne S. Meyer.

(To be submitted to Scientific Report)

Isolation and screening of aerobic marine bacteria from sea cucumber to identify novel fucoidan modifying enzymes

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Abstract

Ninety seven of aerobic marine bacteria that isolated from sea cucumber in Vietnam were used for screening fucoidan-modified enzyme. Fifteen strains showed the activities on fucoidan from *Sargassum mcclurei* and *Turbinaria ornata* by fucoidan-plate method and three bacterial strains produces sulfatase on 5-Bromo-4-chloro-3-indolyl sulfate potassium salt substrate. Those strains were identified by 16S RNA. *Pseudolalteromonas* sp MB47, *Cobetia* sp MB87 was showed the highest sulfatase and fucoidanase activity. The endo fucoidanase activities of those strain were confirm by C-PAGE method.

INTRODUCTION

Fucoidans are a mixture of sulfated fucose-containing polysaccharides, which is present in the cell wall of brown seaweeds (Usov and Bilan 2009) but have also been found in several sea cucumbers (Yu et al. 2014) and sea urchins (Mulloy et al. 1994). Recently fucoidan was furthermore found in some sea grasses. The structure of fucoidans is complex due to varieties of monosaccharide composition, branching as well as differences in glycosidic linkages and in sulfation and, acetylation patterns. The monosaccharides found in fucoidan besides fucose includes mannose, glucuronic acid, xylose, and other more rare monosaccharides like rhamnose (Kusaykin et al. 2008). The position of the sulfate groups is contributing to the complexity of the fucoidan structure. Fucoidan prepared from *Fucus vesiculosus* is commercially from Sigma content 26.3% sulfate and sulfate group are occupied at C2, C2/C3, C2/C4, C4 of L-fucosyl residues (Holtkamp 2009) (Chizhov et al. 1999) (Holtkamp et al. 2009). The fucoidan from *Fucus evanescens* has a sulfate substituted at C2. Additional sulfate occupies position 4 in some of the $\alpha(1\rightarrow3)$ -linked fucosyls, whereas a part of the remaining hydroxyl groups is randomly acetylated (Bilan et al. 2002). The *S. mcclurei* fucoidan is essentially a sulfated galactofucan polysaccharide with 34% sulfate and they differentially sulfated at C2 and/or at C4 and some of the galactosyl moieties are even sulfated at C6 (Pham et al. 2013). Fucoidan extracted from *T. ornata* that were collected at Nha-Trang bay, Vietnam, high sulfate content of ~25% with the sulfate attached mostly at C2, and to lower degree at C4, of both the fucosyl and the galactosyl residues (Thanh et al. 2013) (Ermakova et al. 2015).

Fucoidans have attracted a lot of attention due to the diverse biological activities found and because of the low toxicity, and plant origin (Berteau and Mulloy 2003), (Kusaykin et al. 2008). Fucoidans were reported to have various biological effects in vitro and in vivo such as anti-inflammatory, anticoagulant, antithrombotic (Kusaykin et al. 2008), (Cumashi et al. 2007), antiviral including anti-HIV (Lee et al. 2004) (Thuy et al. 2015), immunomodulatory (Raghavendran, Srinivasan, and Rekha 2011), antioxidant (Jing Wanga et al. 2008), and antitumor (Zhuang et al. 1995) (Aleksyenko et al. 2007). The bioactivity depend not only on the size of fucoidan molecular (Morya, Kim, and Kim 2012) but also the sulfate group (Qiu, Amarasekara, and Doctor 2006). Until now, fucoidan which have high molecular weight (from 13kda to 930kda), high viscosity and complex structure have not yet been applied in medicinal. So people try to create the biologically active fragments of sulfated fucooligosaccharides and also study the relationship of their bioactive and the structure. So enzymes with known specificity that catalyze fucoidan hydrolysis (fucoidan hydrolases, sulfatases) are the best tool to do it without changing their native structure.

In marine ecosystems, fucoidan is a rich carbon source for different organisms, and is expected to be degraded by these organisms, such as invertebrates and bacteria and fungi. Fucoidan-degrading enzymes are produced by algal feeders such *Lambis* sp. (Silchenko et al. 2014). They are also produced by bacteria associated with brown seaweeds, as well as sea cucumber and sea urchin (S. Furukawa et al. 1992) (Bakunina et al. 2000) (Sakai, Kawai, and Kato 2004). Some fucoidan-degrading enzymes have been well characterized and furthermore been cloned and expressed, such as the fucanase FcnA from *Mariniflexile fucanivorans* (Colin et al. 2006) and the two sulfated fucan hydrolases, Fda1 and Fda2, of *Altermonas* sp. SN-1009 (Takayama et al. 2002). However, to date, no fucoidan sulfatase has been identified.

Sea urchins (Echinoidea) and abalones (Gastropoda) mainly ingest algae, with a preference for brown algae. Proteobacteria and Bacteroidetes associated with sea cucumber were reported to producers of fucoidanases (Bakunina et al. 2000). To modified fucoidan from *S.mcclurei* and *T.ornata* in Vietnam Sea, we expect to find enzymes from sea cucumber that live in this sea region. On the other hand, the envelope of sea cucumbers contains fucoidans; therefore, the associated bacteria may possess enzymes capable of degrading these polysaccharides.

So in this study, we will report the isolation and screening of aerobic marine bacteria from sea cucumber for producing fucoidan modified enzymes in Vietnam Sea. Ninety five of aerobic marine bacteria was isolated from gut sea cucumber that sampled in Nhatrang Bay, Vietnam. Fifteen strains from them produced fucoidan modified enzymes. *Cobetia* sp MB87 produced the endo-fucoidanase and sulfatase enzymes and *Pseudoalteromonas* species MB47, *Pseudoalteromonas* species MB104 produced sulfatase.

MATERIAL AND METHODS

Preparation and composition analysis of fucoidan.

Crude fucoidans from *Sargassum mcclurei* and *Turbinaria ornata* was obtained as described (Zvyagintseva et al. 2003). Total carbohydrates were determined by the phenol-H₂SO₄ method using fucose as standard. Sulfate content was measured using gelatin/BaCl₂ method after hydrolysis in 1M HCl for 6 hours. Monosaccharide-composition of fucoidan was determined after hydrolysis in 4N TFA (CF₃COOH) at 100°C for 6 hours and measured on a Biotronik Carbonhydrate Analyzer (Durrum-X4-20; 0.63x30 cm; 60°C; Bicinchonitate method; Shimadzu C-R2AX detector). The monosaccharides xylose, rhamnose, mannose, fucose, galactose, glucose were used as standards. Uronic acid content was determined by carbazole method using D-glucuronic acid as a standard (Bitter and Muir 1962).

Isolation of fucoidan-utilizing marine bacterial strains.

The research object was aerobic marine bacterial isolates from sea cucumber, which was samples in May, 2015 in different place in Nha Trang Bay, Vietnam (Table1. The samples were stored on ice and transported to the laboratory within one hour after collection. After the surface skin of sea cucumbers was sterilized with 70% ethanol, the ventral surface was dissected with a sterile scalpel to expose the body cavity. Thereafter, the digestive tract of sea cucumbers was carefully squeezed out into a sterile tube. All gut content and sediment samples were mill with sterile sea water. The mixture then was spread on nutrient media. Nutrient media components for the isolation of bacteria containing (g/l sea water) Difco bactopectone, 5.0; Difco yeast extract 2.0; *Sargassum mcclurei* Fucoidan, 1; K₂HPO₄, 0.2; and MgSO₄, 0.05 (pH 7.5-7.8), 28-30°C. The resulting colony was inoculated into plates with solid medium (without fucoidan) to obtain pure bacterial cultures.

Screening marine bacteria for fucoidan modifying enzymes

The pure bacterial strains was used for screening of fucoidan modifying enzymes by fucoidan-agar plate method (Silchenko et al. 2015). The strains were cultivated for 3 days at 28°C on marine agar 2216 (BD) containing 0.5% fucoidans from *Fucus vesiculosus* (Sigma), *Sargassum mcclurei* and *Turbinaria ornata*. Bacterial cells were removed from the agar surface and a 1% solution of hexadecyltrimethylammonium bromide (cetavlon) in distilled water was added to the Petri dishes.

After incubation for 30 min at 25°C, the dishes were washed with water several times. Transparent areas appeared under colonies possessing fucoidanase activity.

α -L-fucosidases and sulfatases were detected by using 5-bromo-4-chloro-3-indolyl- α -L fucopyranose (X-fuc) and 5-Bromo-4-chloro-3-indolyl sulfate potassium salt (X-SO₄). Substrates were purchased from Carbonsynth (United Kingdom, Compton) and MB solid media were supplemented at concentration of 100µg/ml. The activity of the strains was detected by blue color formation either around the colonies when the enzymes were secreted or blue colonies when the enzymes were intracellular (Beil et al. 1995).

Identification of strains by 16S ribosomal RNA analysis

The selected strains from fucoidan-agar plate were identified based on the sequencing of 16S rRNA ribosomal gene sequence. The biomass from 5ml overnight culture in MB liquid media was collected by centrifugation and genomic DNA of selected strains was isolated by Blood and Tissue DNA kit, following manufacturer's recommendations for Gram-positive bacteria. The 16S ribosomal RNA (rRNA) gene fragment was amplified using The biomass from 5ml overnight culture in MB liquid media was collected by centrifugation and genomic DNA of selected strains was isolated by Blood and Tissue DNA kit, following manufacturer's recommendations for Gram-positive bacteria. The 16S ribosomal RNA (rRNA) gene fragment was amplified using Phusion® High-Fidelity DNA Polymerase (NEB, US) with the universal bacterial primers (533F: 5'-GTGCCAGCAGCCGCGGTAA-3' and 1392R: 5'-GGTTACCTTGTTACGACTT-3'), blunt-end cloned into pJET1.2 vector using CloneJET PCR Cloning Kit (ThermoFisher Scientific,US) and propagated in E. coli DH5alpha strain. After plasmid preparation, inserts were sequenced at Macrogen (Korea) using Sanger capillary sequencing method. Sequences were aligned against 16S ribosomal RNA entries of NCBI RefSeq database in order to identify the taxonomic identity of closest homologues. ng Phusion® High-Fidelity DNA Polymerase (NEB, US) with the universal bacterial primers (533F: 5'-GTGCCAGCAGCCGCGGTAA-3' and 1392R: 5'-GGTTACCTTGTTACGACTT-3'), blunt-end cloned into pJET1.2 vector using CloneJET PCR Cloning Kit (ThermoFisher Scientific,US) and propagated in E. coli DH5alpha strain. After plasmid preparation, inserts were sequenced at Macrogen (Korea) using Sanger capillary sequencing method. Sequences were aligned against 16S ribosomal RNA entries of NCBI RefSeq database in order to identify the taxonomic identity of closest homologues.

Confirmation of fucoidanase and sulfatase activity

Marine bacterial strains for the primary screening of fucoidan-degrading enzymes producers were grown on a shaker (160 rpm) at 28°C for 24h in a medium containing (g/l water) Difco bactopectone, 5.0; Difco yeast extract, 1.0; fucoidan 1.0; sea salts, 28.0 (pH 7.5-7.8). Cells were harvested by centrifugation at 4,500 g for 30 min and 4°C and the pellet was re-suspended in 20 mM Tris-HCl buffer, pH 7.2 buffer before being disrupted by UP 400S Ultrasonic processor with 0.5 cycle and 100% amplitude. Cell debris was pelleted by centrifugation (20,000×g, 20 min at 4°C). The supernatant obtained by centrifugation was then filtered through a 0.45µm filter and was used to test activity of the intracellular enzymes.

The endo-fucoidan activity was confirmed Carbohydrate-Polyacrylamide Gel Electrophoresis (C-PAGE). The reaction mixtures containing 0.5µg/µl enzyme solution in 20mM Tris-HCl buffer pH 7.2, 10mM CaCl₂ and 1% weight/volume

fucoidan in buffer were incubated at 35°C for 24h-48h. Each reaction mixture (10µl) was mixed with 5µL loading buffer (20% glycerol and 0.02% phenol red). Samples (5µL) were electrophoresed at 400V through a 20% (w/v) 1 mm thick resolving polyacrylamide gel with 100mM Tris-borate buffer pH 8.3 for 1h. Gel staining was performed in two steps, first with a solution containing 0.05% alcian blue 8GX (Panreac, Spain) in 2% acetic acid for 45 mins and then with 0.01% O-toluidine blue (Sigma-Aldrich, Steinheim, Germany) in 50% aqueous ethanol and 1% acid acetic.

The uronic acid lyase of crude fucoidan substrate was measured by reducing sugar method. A standard reaction mixture contained a 0.1% substrate solution (200µl) and an enzyme solution (50 µl) in 20mM Tris-HCl buffer pH 7.2. The incubation was carried out for 4 hour at 37°C. The activity during the hydrolysis of polysaccharides was determined from an increase of the amount of reducing sugars by the method of Nelson.

The fucoidan-sulfatase was confirmed by measuring the sulfate release by high performance anion exchange chromatography (HPAEC) using a Dionex ICS-5000 chromatography system equipped with a GP40 gradient pump and an ED40 electrochemical detector. The ions are separated on an HC-AS11 anion-exchange column (4 x 250 mm; Dionex) with accompanying HC-AG11 guard column (4 x 50 mm; Dionex). Elution is done with 35 mM NaOH using an isocratic flow rate of 1.5 mL/min. Background signal and noise originating from the eluent is reduced using an anion self-regenerating suppressor (ARS-500, Dionex) with a current of 180 mA. The suppressor converts the eluent to a weakly dissociated form prior to detection, by removing counter ions and replacing them with regenerated ions. Run-time is 15 min and sulfate elutes separately from other ions at approx. 4 min. Sulfate concentration is deduced from the signal intensity and calculated from a standard sulfate calibration curve using Na₂SO₄.

RESULTS

Preparation and composition analysis of fucoidans

Fucoidan from Vietnamese brown seaweed *S. mcclurei* and *T. ornata* was extracted and described in Table 1. The results indicate that *S. mcclurei* and *T. ornata* fucoidans are sulfated galactose fucans, as expected. The main component of *S. mcclurei* fucoidan is fucose (38.5%) and galactose (32.3%) and *T. ornata* fucoidan are 55.8% fucose and 24.8% galactose. This crude fucoidan was used for screening marine bacteria that ability produce fucoidan modified enzymes for next step by fucoidan-agar plate method.

Table 1. Monosaccharide composition of fucoidan from *S. mcclurei* and *T. ornata* by XX

Fucodian	Uronic acid (% w/w)	SO ₃ Na (% w/w)	Monosaccharide composition (% mol)				
			Fucose	Galactose	Glucose	Mannose	Xylose
<i>S.mcclurei</i>	17.87	33.15	38.5	33.1	20.6	4.2	3.6
<i>S.mcclurei</i> <i>pure</i>	-	35.03	58.5	41.5	-	-	-
<i>T. ornate</i>	7.50	25.30	55.8	24.8	1.2	9.2	9.0

(-) not detected

Isolation and screening of marine bacteria of fucoidan modifying enzymes

From fifteen sea cucumber samples that collected in at different position Nha Trang Bay, Vietnam, there were 97 strains of aerobic bacteria were isolated. Isolated strains of bacteria have different colony characteristics, so we presume that they are different strains of bacteria. From 97 aerobic bacteria strains that isolated from sea cucumber in Vietnam Sea, 15 strains showed the activities on fucoidan from *S. mcclurei* and *T. ornata* by fucoidan agar- plates method (Figure 1). The results showed that 15 strains made the disappearance of fucoidan from agar plate. MB 87 showed the highest activity on both cure fucoidan from *S. mcclurei* and *T. ornata*. The disappearance of fucoidan occurs because in the bacterium present some enzymatic apparatus able to catabolize fucoidan such as fucoidanase, sulfatase and uronic acid lyase. The identification of 15 selected strains was conducted by 16S rDNA; the results are presented in Table 2.

Table 2. Fucoidan modifying activities of isolated marine bacteria from the gut of sea cucumber

Code	Closest relatives (by 16S rDNA)	Fucoidan – plate method		Sulfatase
		<i>S. mcclurei</i> fucoidan	<i>T. ornata</i> fucoidan	<i>X-SO₄</i>
MB 03	<i>Pseudomonas sp.</i>	+	+	-
MB 04	<i>Pseudomonas sp.</i>	+	+	-
MB 05	<i>Ancinerbacter baumanii</i>	+	+	-
MB 06	<i>Pseudomonas sp.</i>	+	+	-
MB 09	<i>Ocianomonas sp</i>	+	+	-
MB 26	<i>Bacillus sp.</i>	+	+	-
MB 31	<i>Shawannella sp.</i>	+	+	-
MB 32	<i>Shawannella sp.</i>	+	+	-
MB 47	<i>Pseudoalteromonas sp.</i>	+	+	+++
MB 87	<i>Cobetia sp.</i>	+++	+	+
MB 95	<i>Bacillus aryabhattai</i>	+	+	-
MB 96	<i>Bacillus sp.</i>	+	-	-
MB 104	<i>Pseudoalteromonas sp.</i>	+	+	+
MB 109	<i>Photobactenia sp</i>	+	-	-
MB 110	<i>Photobactenia sp</i>	+	-	-

(-) no activity

(+) have activity

(+++) Very high activity

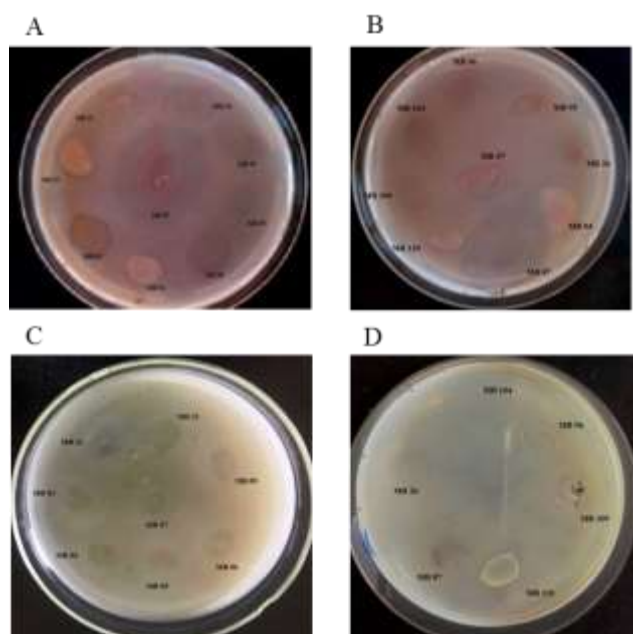


Figure 1. Fucoidan-agar plate method of detection of fucoidan-modifying enzymes of bacteria with using solid medium with *S. mcclurei* (A, B) and *Turbinaria ornata* (C) fucoidan treated with a 1% aqueous solution of cetavlon.

Confirmation of fucoidan modifying ability

To confirm the fucoidanase activity, the C-PAGE which the most frequent method used for analyzing fucoidan degradation by enzymes was used. Treatment of two different pure fucoidan samples from *S. mcclurei* and *T. ornata* with the individual intracellular of 15 selected marine bacterial strain produced different C-PAGE electrophoresis patterns with the 2 fucoidan samples. However, only *Cobetia* sp MB87 was seen to have positive activity on both fucoidan from *S. mcclurei* and *T. ornata* (Fig.2). In general, the data obtained showed that all the enzymes produced differently sized sulfated oligomers in the C-PAGE chromatograms, suggesting that the different enzymes target different linkages and/or differently sulfated fucosyl residues. Furthermore results also suggest that all investigated enzymes were endo-acting. The enzymatic action left behind relatively high molecular weight fractions.

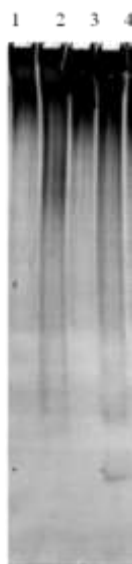


Fig 2. C-PAGE product profiles of fucoidan degradation using crude enzymes (1) *S. mcclurei* fucoidan, (2) enzymatic reaction of intracellular MB87 and *S. mcclurei* fucoidan, (3) *T. ornara* fucoidan, (4) enzymatic reaction of intracellular MB87 and *S. mcclurei* fucoidan.

The sulfatase and α -L-fucosidase activities were performed on X-SO₄ and X-Fuc agar plate. However, there is no strain active on X-SO₄ plate. *Pseudoalteromonas* sp. MB47, *Cobetia* sp. MB87 and *Pseudoalteromonas* MB104 bacterial strains have the positive signal on X-SO₄ plate. These colonies turn to blue after 3 days incubated at 30°C (Fig 3). *Pseudoalteromonas* sp. MB47 showed the strongest blue colour suggesting intracellular sulfatase activity, which was verified when testing the crude extracts (Fig 3B).

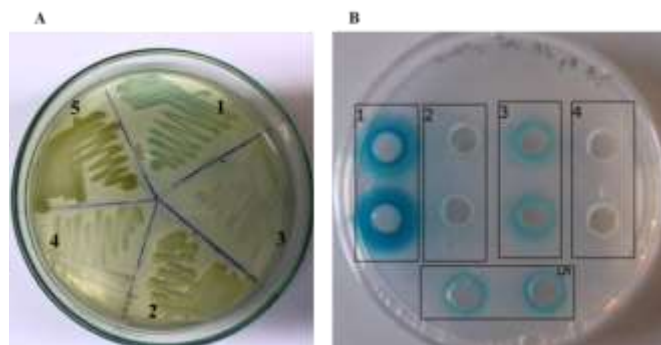


Fig 3. Sulfatase activity on X-SO₄ plate. A) *Pseudoalteromonas* sp. MB47 colony; B1) crude extract from *Pseudoalteromonas* sp. MB47; B2) crude extract from *Pseudoalteromonas* sp. MB06; B3) crude extract from *Cobetia* sp. MB87; B4) crude extract from *Pseudoalteromonas* sp. MB04; B5) crude extract from *Pseudoalteromonas* sp. MB104

DISCUSSION AND CONCLUSION

Sargassum mcclurei and *Turbinara ornata* distribute the most in Nhatrang Bay, Vietnam (Fig S2) (Bui Minh Ly, et al 2010). In the sea region, sea cucumber the algae feeders which are the traditional nutritional food and medicinal resource widely consumed in Vietnam are abundant. So sea cucumber or their associated bacteria might produce enzyme system capable to degrading algal cell-wall polysaccharide to mono-or oligosaccharides. For this reason, screening fucoidan modified enzymes was primary carried out from gut sea cucumber bacteria. Ninety seven of marine bacteria isolated from gut sea cucumber by BM media content fucoidan as carbon source was dominated for this hypothesis. In this study, we used crude fucoidan from *S.mcclurei* as carbon source which contain fucose, galactase, manose, xylose, uronic acid and sulfate (Table 1). For transformation this fucoidan, a complex of various enzymes (fucoidanases, sulfatases, fucosidases, fucose permease, galactosidases etc) capable of transforming fucoidan and transporting the products of its cleavage into the cell of bacteria can be present in the bacteria

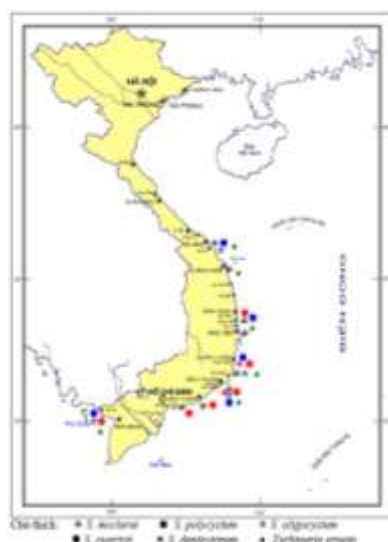


Fig S2. The distribution of fucoidan in Vietnam sea

Fucoidanase activity can be detected by a viscometric assay specific for endodepolymerases (Kitamura et al., 1992; Furukawa et al., 1992), measurement of an increase in the content of reducing end groups (Ivanova et al., 2003; Bakunina et al., 2000), or by a carbohydrate–polyacrylamide gel electrophoresis (C–PAGE) assay based on the release of anionic oligosaccharides from sulfated fucan (Colin et al., 2006). However, these assays still have these disadvantages such as large amount of substrate and enzymes, expensive, time consuming and difficult to apply to screening large amount of samples. In this study we used a method for the rapid screening of a large number of bacterial strains-fucoidan agar plate. This simple method are used for the determination fucoidan utilization by bacteria growing on agar medium. This method is based on the ability of the cationic detergent hexadecyltrimethylammonium bromide (cetavlon) to form a water-insoluble salt with a white color in the presence of fucoidan. By this method we saw 15 marine bacterial strains had positive signal on fucoidan from *S. mcclurei* and *T.ornata*. The disappearance of fucoidan from agar plate occurs because in the bacterium present some

enzymatic apparatus able to catabolize this class of polysaccharide. It is impossible to associate the disappearance of fucoidan with identification of a single enzyme (sulfatase or fucoidanase). The results indicate the nutritional importance of fucoidans for the bacterial assassins of sea cucumber. The fucoidan-degrading ability may be part of the adaptive response of microorganisms colonizing various marine animals to the environment, through which these microorganisms satisfy their requirements for carbon, sulfur, and energy.

On other hand, the fucoidan preparations contain uronic acids 17.87 % of *S. mcclurei* and 7.50% of *T. ornata*. Uronic acids have previously been found in the branches of fucoidans but it can also be alginic acid contamination from the fucoidan purification procedure. The amount of these compositions did not interfere with agar-plate method. Addition, the enzymatic reaction of total extract from those strain and fucoidan did not showed any activity by reducing sugar method, so they might not produce alginic acid lyase. However, the fucoidanase activity need to confirm by the C-PAGE.

By C-PAGE method, there was just *Cobetia* sp was show the activity on both pure fucoidan from *S.mcclurei* and *T.ornata* (Figure 2). The data indicated more profound degradation of the fucoidan substrates from *T. ornata* (4) than *S. mcclurei* (2). Both fucoidan substrates predominantly have α (1→3) glycoside bonds in their backbone structures. However, *S. mcclurei* produce sulfated galactofucan polysaccharides having both α (1→3) and α (1→4) linked fucosyl residues, differentially sulfated at C2 and/or at C4 (Pham et al. 2013), while the backbone of *T. ornata* fucoidan has been proposed to solely consist of α (1→3)-linked L-fucosyls with the sulfate attached mostly at C2, and to lower degree at C4 (Thanh et al. 2013) (Ermakova et al. 2015). Thus, the results suggest that *Cobetia* sp. MB87 utilize fucoidan by degrading it and that the fucoidan – degrading enzyme was produced in side those cells. There are several reports describing marine bacteria producing fucoidanases such as *Vibrio* sp. No.5, *P. citrea* KMM 3296, KMM 3297, KMM 3298, *S. paucimobilis* PF-1, *Flavobacterium* sp. SA-0082, *Alteramonas* sp. SN-1009, *M. fucanivorans* SW5, *Formosa algae* KMM 3553. However, this is the first evidence that *Cobetia* sp. produce fucoidan degrading enzymes.

The abundance of fucoidan-degrading marine bacteria and their low fucoidanase activity may be due to the specific role of fucoidans and fucoidanases in the metabolism of marine organisms. Fucoidan modifying enzymes were produced by associated bacteria from sea cucumber gut, although their apparent activity was low. Because of unstable nature fucoidanase, they degrades themselves when it release out of marine microorganism (Cao et al. 2018) and then they lost the activity very quickly. Moreover, in the procced to purify fucoidanase, the activity was lost very quickly. Although the first fucoidanase was studies several decades ago, there are just 10 known fucoidanase sequences of fucoidanases in the CaZy database (Lombard et al. 2014) (included fucoidanases FFA1 and FFA2 from marine bacterium *Formosa algae*) and all of them belong to the phylum of Bacteroidetes. However, in our study the selected bacteria isolated from sea cucumber gut include *Pseudomonas* sp., *Ancinerbacter baumannii*, *Ocianomonas* sp., *Bacillus* sp., *Shawannella* sp., *Photobactenia* sp are all belong to the gamma-proteobacteria (Table 2). These results share with previous publication that members of γ -proteobacteria were the predominant bacterial group in the gut contents and surrounding sediment of the sampled sea cucumbers that was reported previously (Gao et al. 2014), unculturable γ -proteobacteria were found predominant in gut contents of sea cucumbers cultured under the pond culture mode (Gao et al., 2010) and culturable γ -proteobacteria (*Vibrio*, *Pseudomonas*) were found dominant in the digestive tract of *A. japonicus* (Sun and Chen, 1989). Analysis the sequence of

Cobetia sp., there is no sequences are present in *Cobetia* sp that look like the Bacteroidetes sequences and it could therefore be a completely new kind of fucoidanase. Thus, the analysis the genome of this *Cobetia* sp strain might be giving an idea of gene coding for fucoidanase.

In our study, there are two *Pseudoalteromonas* species MB47 and MB104, which both seem to produce sulfatases. *Pseudoalteromonas* was previously reported to produce a carrageenan sulfatase (Genicot et al. 2014). The desulfatation of fucoidan from *S. mcclurei* and *T. ornata* by the two *Pseudoalteromonas* sp. species MB47 and MB104 as well as the *Cobetia* sp. MB87 was however not successful. This might be due to the sulfatases are only capable of hydrolyzing small fucoidan oligosaccharide rather than the natural fucoidan polymers. This has been reported previously, in the hepatopancreas of marine mollusks *Haliotis* sp., *L. kurila* (Kusaikin et al. 2006). The sulfatase from the marine bacteria *Vibrio* sp. N-5 eliminates sulfate from sulfated fucose and short fuco-oligosaccharides released after fucoidanase hydrolysis, while the activity towards the native fucoidan was very limited (Furukawa et al. 1992). A partially purified preparation enzyme from the bivalve mollusk *P. maximus* effectively desulfatated the synthetic substrate p-nitrocatecholsulfate, L-fucose sulfated at C2 and a natural fucoidan polymer from *A. nodosum* of 13kDa.

In summary, from screening of 97 marine bacterial strains from gut sea cucumber in Nhatrang Bay, Vietnam, *Cobetia* sp MB87 produced the endo-fucoidanase and sulfatase enzymes and *Pseudoalteromonas* species MB47, *Pseudoalteromonas* species MB104 produced sulfatase, these enzyme works on synthesis substrates.

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A Thermostable Fucoidan Active Sulfatase

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Abstract

Marine polysaccharides are very abundant and very complex molecules found in the oceans. Many marine polysaccharides are highly sulfated including carrageenans, agarans and fucoidans found in the cell walls of macro algae. These sulfated polysaccharides are an important food source for heterotrophic organisms and requires a set of degradative enzymes to be utilized. These include glycosyl hydrolases to cleave the linkages of the carbohydrate backbones and sulfatases to release the sulfate groups.

We report the first findings of a fucoidan active arylsulfatase (ARSF1). This sulfatase was found in the sea cucumber gut bacteria *Pseudoalteromonas* sp. The sulfatase belongs to the sulfatase I family of arylsulfatases. We found that the sulfatase requires Ca^{2+} for function and an observed optimal pH of 6.5 and a surprisingly high optimal temperature of 68°C. The K_M and V_{\max} of the sulfatase were determined to be 0.95mM and 26.6μM per minute respectively on the p-nitrocatechol sulfate substrate (pNCS). The sulfatase was able to release sulfate from fucoidan oligosaccharides obtained after degradation of the fucoidan from the brown algae *Sargassum mcclurei* using different fucoidanases. ARSF1 is hereby the first characterized fucoidan-acting sulfatase.

Keywords: arylsulfatase, ARSF1, Pseudoalteromonas, thermostable, sea cucumber, bacterial, polysaccharide, fucoidan, fucoidanase, brown seaweed.

Introduction

Fucoidan is a highly sulfated polysaccharide found in the brown algal cell wall. The structure of fucoidans is very complex and only a few fucoidan oligosaccharides have been determined in detail.

The backbone of fucoidans mainly consists of α -D-fucose residues linked by either 1-3 or 1-4 glycosidic linkages. In some species of brown algae the fucoidans have up to a 1:1 ratio of fucose: galactose in the backbone. These galactofucans have been found in several species of brown algae, including *Sargassum mcclurei* and *Turbinaria Ornata*, from the Vietnamese sea (Thin *et al.*, 2013; Thanh *et al.*, 2013).

The fucose and galactose residues can be sulfated at different carbons dependent on the species. In *Fucus evanescens* the prevalence is on C2 and sometimes C4 sulfations (Bilan *et al.*, 2002), while fucoidans from *Fucus vesiculosus* have been found with many different sulfation patterns including sulfates on C2, C2/C3, C2/C4 or C4 (Holtkamp 2009, Chizhov *et al.*, 1999, Holtkamp *et al.*, 2009). The fucoidan from *U. pinnatifida* is moreover assumed to be rich in 2, 4-disulfate substitutions (Anastyuk *et al.*, 2009). *S. mcclurei* fucoidan is sulfated at C2 and/or at C4 and some of the galactosyl moieties are sulfated at C6 (Thin *et al.*, 2013).

The fucoidans can furthermore be branched, which contributes considerably to the complexity of the molecule. The high complexity of fucoidans likely requires a sophisticated array of degradative enzymes in the organisms living on these polymers (Michel and Czjzek, 2013). Not only do they need to produce different glycosyl hydrolases to catalyze the cleavages of the different glycosyl linkages, they also need sulfatases specific for the differently sulfated fucose and galactose residues.

A few fucoidanases have been described in literature, most of which catalyze the cleavage of α -1-4 fucosyl linkages, but also two have been described to cleave α -1-3 linkages, while no fucoidan degrading enzyme have been found cleaving the fucose-galactose linkages to date.

Although fucoidan sulfatase activity has previously been described, no sequences have yet been determined (Cock *et al.*, 2010). Sulfatases active on polymeric fucoidan substrates or oligosaccharides have been reported, pointing at the unexplored diversity of fucoidan sulfatases (Daniel *et al.*, 2001).

Sulfatases represent a large family of enzymes that have been divided into three classes based on homology, crystal structures and mechanisms. Polysaccharide sulfatases characterized to date, all belong to Type I sulfatases (S1), which are formyl-glycine-dependent sulfatases. They catalyze the cleavage of sulfate ester groups with a hydrolytic mechanism (EC 3.1.6.-sulfuric ester hydrolases; EC 3.10.1.- sulfamidases). S1 sulfatases include the vast majority of the characterized sulfatases to date (Hanson *et al.*, 2014). These sulfatases require an essential post-translational modification of the conserved catalytic site cysteine or serine into a formyl-glycine.

The formation of the formyl-glycine is catalyzed by another enzyme called the formyl-glycine-generating enzyme (FGE), which recognizes the conserved N-terminal motif [C/S]XPXR (Cosma *et al.*, 2003; Dierks *et al.*, 2003; Sardiello *et al.*, 2005; Bojarová and Williams, 2008; Schmidt *et al.* 1995; Miech *et al.* 1998). The FGly formation is thought to occur post-

translationally and before the sulfatases are folded, which is thought to occur after cell excretion (Appel and Bertozzi, 2015).

Although no fucoidan sulfatase sequence has yet been described, a few marine polysaccharide specific sulfatases have been characterized, including agar and carrageenan sulfatases. These sulfatases have all been derived from marine bacteria. The carrageenolytic strain *Pseudoalteromonas atlantica* T6c produce a Type I sulfate enzyme that catalyzes the specific removal of the sulfate at position 4 of the β -linked galactose in ι -carrageenan, resulting in the conversion of ι -carrabiose into α -carrabiose repetition units (Préchoux *et al.*, 2013; Préchoux and Helbert, 2014). The gene encoding the sulfatase has been described and expressed recombinantly in *Escherichia coli*. In contrast to this exo-acting sulfatase, an endo-acting sulfatase was found in *Pseudoalteromonas atlantica* T6c, which catalyzes the desulfation of κ -carrabiose, resulting in a neutral β -carrabiose repetition moiety.

Desulfation of agars has been shown in several marine bacterial strains including *P. carrageenovora* (Kim *et al.*, 2005), *Flammeovirga pacifica* (Gao *et al.*, 2015), *Pyrococcus furiosus* (Jung *et al.*, 2012) and *Thermotoga marina* (Lee *et al.*, 2013). Although the position of the sulfate group cleaved has not been determined, all these sulfatases were characterized as Type I sulfatases.

A previously characterized protein Psc ι -CgsA (AER35705) from *Pseudoalteromonas carrageenovora* strain PscT was found to catalyzes the hydrolysis of the 4-sulfate of ι -carrageenan (Genicot *et al.*, 2014) (Kim *et al.*, 2005). This sulfatase was the first member of a new sulfatase family, with a C-terminal domain having strong similarity with the superfamily of amidohydrolases.

However, reports on true fucoidan sulfatases are to date very limited. A sulfatase from marine bacteria *Vibrio* sp. N-5 eliminates sulfate from sulfated fucose and short fucooligosaccharides formed by fucoidanases, since its activity towards native fucoidan was very limited (Furukawa *et al.*, 1992). A partially purified preparation from the bivalve mollusk *Patinopecten maximus* effectively desulfated a synthetic substrate p-nitrocatechol sulfate (pNCS), L-fucose sulfated at C2 and a natural fucoidan from *A. nodosum* of 13kDa. However, calculations have shown that only approximately 10% of the sulfate content was removed from the fucoidan sample containing 34% of sulfate substituent (Sasaki *et al.*, 1996). Sulfatase hydrolyzing p-nitrophenyl sulfate (pNPS) rather than natural fucoidan was found in the hepatopancreas of marine mollusks *Haliotis* sp., *L. kurila* (Kusaikin *et al.*, 2006).

Previously, in the attempt to find novel fucoidan modifying enzymes, sea cucumbers were collected along the Vietnamese coast and several gut bacteria were isolated and screened for activity (Cao *et al.* 2018). A bacterial strain with apparent high sulfatase activity was isolated and the bacterium was determined through 16S analysis as *Pseudoalteromonas* sp..

We report the first findings of a fucoidan active arylsulfatase (ARSF1). This sulfatase was found in the sea cucumber gut bacteria *Pseudoalteromonas* sp. The sulfatase belongs to the sulfatase I family of arylsulfatases. We find that the sulfatase requires Ca^{2+} for function and has an observed optimal pH of 6.5 and a surprisingly high optimal temperature of 68°C. The K_M and V_{max} of the sulfatase was determined to be 0.95mM and 26.6 μ M per minute respectively on the pNCS substrate.

Interestingly, the sulfatase was able to release sulfate from fucoidan oligosaccharides obtained after degradation of the fucoidan with fucoidanases the brown algae *Sargassum mcclurei* and is the first characterized fucoidan acting sulfatase.

Results

Identification of sulfatases in the genome of Pseudoalteromonas sp.

In total 7 putative protein sequences of *Pseudoalteromonas* sp. MB47 were annotated with InterPro sulfatase signatures. All of them contained sulfatase domain signature IPR000917. 5 of these proteins contained this domain at the N-terminal of the protein and exhibited high similarity to proteins annotated as arylsulfatases in *Pseudoalteromonas lipolytica* when compared using blastp against nr database. The remaining 2 proteins were annotated as sulfatase-domain containing lipoteichoic acid synthase and phosphoethanolamine transferase. Based on blastp comparison with SulfAtlas database (v1.2), arylsulfatases can be classified as belonging to subfamily S1_4 (IMPJCBKJ_00303: ARSUL2) and S1_13 (IMPJCBKJ_00307: ARSF1, IMPJCBKJ_00492: ARSUL3, IMPJCBKJ_02557: ARSUL4 and IMPJCBKJ_022757: ARSUL1; Fig S5). While S1_4 contains a number of characterized proteins with arylsulfatase activity (EC 3.1.6.1), the specificity of S1_13 subfamily is unknown. Additionally, analysis of ARSUL2 and ARSF1 revealed sulfatase conserved sites, described by ProSitePatterns PS00523 and PS00149, respectively (Table S2).

Five putative arylsulfatases ARSUL1, ARSUL2, ARSF1, ARSUL3 and ARSUL4 were predicted by sequence homology. The sulfatases belong to the arylsulfatase family S1 according to SulfAtlas (<http://abims.sb-roscoff.fr/sulfatlas/index.html?execution=e3s1>, Barbeyron *et al.*, 2016). The sulfatase sequences from *Pseudoalteromonas* sp. contain predicted signal peptides in the N-terminals and they all contain the conserved active site (CTAGRAALITG an 11 amino acid stretch in the active site), which includes the conserved cysteine (which is post-translationally converted to a formylglycine). Phylogenetic analysis was performed with the predicted *Pseudoalteromonas* sp. sulfatases, to determine the relationship to other known sulfatases (Fig. 1).

ARSUL2 is relatively closely related to ARS (previously AtsA; P51691) from *Pseudomonas aeruginosa* PAO1 (identity 30%), which has been characterized and is active on pNCS substrate and the crystal structure has also been solved (Beil *et al.*, 1995, Boltes *et al.*, 2001), ARSUL3, ARSUL4 and ARSUL1 are closely related to Ary423 from *Flammeovirga pacifica* (identity 51%, 47, 46% respectively), which is active on p-nitrophenyl sulfate (pNPS) and crude asparagus cell wall extracts (Gao *et al.*, 2015), ARSF1 is related to an *E. coli* sequence, which has not yet been characterized (identity of 38% and 29% to Ary423). By BLAST, a hit called Ps3148 was furthermore found in the *Pseudoalteromonas* sp. genome, which is closely related to Psc t-CgsA (85% identity) (Genicot *et al.*, 2014).

Screening for sulfatase activity on X-SO₄ plates

The sulfatases were codon optimized for *E. coli* and synthesized without the N-terminal excretion peptide. They were transformed into *E. coli* and the cells were streaked on 5-Bromo-4-chloro-3-indolyl sulfate potassium salt (X-SO₄) containing LB plates and screened for sulfatase activity at 37°C. The *E. coli* cells with the sulfatase AtsA produced blue colonies, suggesting that the sulfatase was expressed and active on X-SO₄ (Fig. S1A).

Heterologous expression and purification of the recombinant ARSF1 sulfatase

ARSF1 was expressed in different *E. coli* strains by IPTG induction. The optimal expression host used was BL21 (DE3) with the pGro7 chaperone. The sulfatase activity was tested on *E. coli* lysate after sonication on plates containing the X-SO₄ substrate. The positive control ARS had activity on X-SO₄ substrate as expected and ARSF1 also showed activity on X-SO₄, although to lesser extent than ARS.

The ARSF1 sulfatase was purified and desalted. The purified protein was highly pure and had the expected molecular weight of approximately 57 kDa (Fig. 2). The yield of ARSF1 was surprisingly high, up to 57% of the total protein in the *E. coli* lysate.

Characterization of the recombinant ARSF1 sulfatase

Having successfully achieved the recombinant expression and purification of the sulfatase Ps1594 as a soluble enzyme, the sulfatase was further investigated with regards to 3D structure as well as reaction conditions for optimal enzyme activity *in vitro*. These parameters included substrate prevalence, divalent metal ion dependence, pH, temperature optimum, thermostability as well as K_m and V_{max} on the pNCS substrate.

3D structure modeling and substrate docking

The 3D homology model of ARSF1 was inspected visually in PyMOL and its quality was checked using the QMEAN4 Z-score. The model showed a dimeric structure and the expected β -sandwich fold (Fig. 3). In both monomers a calcium ion was bound in the active site, therefore the model suggested a calcium dependent sulfatase mechanism. This finding is also supported by the experimental results as discussed below. The quality of the model with a Z-score of -1.61 was considered to be satisfactory to study also docking of substrates. As molecules for the docking studies three different sulfated fucoses (fucose-2-sulfate, fucose-4-sulfate and fucose-2,4-disulfate) as well as the aromatic model substrates pNPS and pNCS were chosen. As depicted in Fig. 4 and 5 all substrates are mainly bound by three residues close to the active site (H281, N338 and K354) as indicated by the predicted polar contacts. Since the differences in binding modes of the sulfated fucoses were not very pronounced we hypothesize that theoretically all three monomeric molecules could be a substrate for ARSF1 (Fig. 4). Oligomeric or polymeric substrates (*e.g.* fucoidan oligosaccharides from fucoidanase degradation) might be differently positioned in the active site assisted by amino acid residues along the binding subsites, which might give rise to a preferred sulfation side in the natural substrate. The docking experiments also gave no hints for the preferred hydrolysis of pNCS over pNPS. For both substrates productive binding modes could be identified (Fig. 5). From the 3D modelling, it seems highly likely that the sulfatase needs a divalent cation as a cofactor, likely calcium.

The sulfatase ARSF1 is calcium dependent

The influence of different divalent cations on the enzyme activity was determined for ARSF1 by pre-incubation with different concentrations of CaCl₂, MgCl₂, MnCl₂, NiCl₂, CuCl₂, ZnCl₂, and FeCl₂ and the activity on pNCS was subsequently determined (Table 1). The sulfatase was also screened on another low molecular weight sulfated substrate,

pNPS. The sulfatase was also active on the pNPS, but showed prevalence towards the pNCS substrate, although this could not be determined from the 3D model.

The heavy metals Ni^{2+} , Zn^{2+} , Cu^{2+} , and Fe^{2+} were found to have an inhibitory effect on ARSF1 sulfatase activity and resulted in reduced activity of 16, 6, 35 and 8% respectively at 2mM concentrations. The addition of CaCl_2 and MgCl_2 , (up to 10mM) increased the sulfatase activity to 608% and 127% respectively, while MnCl_2 at a concentration of 2mM resulted in an increased activity of 131%. Since the sulfatase activity was increased approximately six times with addition of 10mM Ca^{2+} , it indicated that Ca^{2+} is required for optimal activity, like was suggested by the 3D model.

The pH and temperature optimum of the sulfatase

The effect of pH on sulfatase activity was from 5.0 to 8.0 with pNCS and 10mM CaCl_2 . The enzyme exhibited activity at a narrow pH range from 6.0 and 7.0 (Fig. 6), while the optimal pH of ARSF1 was determined to be 6.5 and the enzyme was essentially inactive at the outlying pH values of 5.0 and 8.0. The temperature dependence of ARSF1 activity was monitored by performing incubations with pNCS at 30, 45, 60, 68 and 75°C (at pH 6.5 and 10mM CaCl_2). ARSF1 was active at a wide temperature range from 35-80°C and the optimal temperature was found to be 68°C (Fig. 6).

ARSF1 showed a high thermostability

The thermostability of the recombinant ARSF1 was evaluated by measuring the residual enzyme activity after pre-incubation with the enzyme at 68°C for various time durations with or without NaCl (Fig. 7). The sulfatase activity was very slowly decreased during the 2 hours of pre-incubation at 68°C and retained as much as 80% activity after 2 hours. This showed that the sulfatase was highly stable at high temperatures for a long time.

When plotting Ln (residual specific activity) vs. incubation time, a linear relationship was observed showing that the ARSF1 sulfatase follows a first order deactivation kinetic after 1 hour of pre-incubation (without NaCl) and 2 hour (125mM NaCl in reaction mixture). Based on the slope obtained, it was possible to calculate the first-order rate constant of the thermal denaturation (KD) at 68°C (Fig. S3). The half-life ($T_{1/2}$: the time it takes for the enzyme activity to be reduced to half) was calculated as $\text{Ln}2/\text{KD}$. The $T_{1/2}$ of ARSF1 at 68°C was 12h (0mM NaCl) and 8h (125mM NaCl), respectively. These results indicate that ARSF1 is more stable in the presence of salt than without.

Desulfation kinetic of ARSF1 sulfatase

Michaelis-Menten kinetics were performed for the ARSF1 sulfatase at 68°C, at pH 6.5, 10mM CaCl_2 , using pNCS as substrate in the presence of 125mM NaCl and in the absence of NaCl (control), following the pNCS formation via absorption at 515nm. The results for the non-linear least squares fit of the Michaelis-Menten model to the measured data are summarized in Fig. S4. The fit parameters in the absence of NaCl gave a K_M value of 0.95 ± 0.15 mM pNCS and a V_{max} of 26.6 ± 1.2 μM pNCS product formed per minute. In the presence of 125mM NaCl, K_M does not change significantly (0.88 ± 0.25 mM) compared to the K_M in the absence of salt. However, a significant effect can be seen on V_{max} (19.9 ± 1.6 μM), where the addition of salt leads to a 25% decrease in V_{max} when compared with the value in the absence of salt. This suggests that within the tested concentration range, NaCl does not hinder substrate binding, but rather influences the

catalytic step and thus acts like a non-competitive inhibitor, i.e. where NaCl can bind both to the free enzyme but also the enzyme-substrate complex, hereby hindering product formation.

The sulfatase ARSF1 desulfates fucoidan oligosaccharides

Purified fucoidan from *S. mcclurei* (Thin *et al.*, 2013) and *F. evanescens* were used for desulfation by the ARSF1 sulfatase. The fucoidan was first degraded by the different fucoidanases, FcnA2Δ229, Fda2 and FdlB. The specificity of these fucoidanases have previously been shown to be different, e.g. α -1,4 fucose linkages, α -1,3 fucose linkages and fucoglucoronomannan lyase activity respectively (Takayama *et al.*, 2002). Interestingly all these fucoidanases have been shown to be active on fucoidan from *S. mcclurei* and *F. evanescens*, releasing fuco-oligosaccharides of different sizes (Cao *et al.* 2018). This would give the largest array of different oligosaccharide products to use for the desulfation with ARSF1.

ARSF1 was found not to be able to release sulfate from the native polysaccharide fucoidans, but was able to release sulfate from several of the produced oligosaccharides after fucoidanase treatment (Table 2). The sulfate release was measured by HPLC and the amount of released sulfate was correlated with the amount of sulfate present in the polysaccharide substrate, which were 35% and 33% for *S. mcclurei* (Thinh *et al.*, 2013) and *F. evanescens* (Bilan *et al.*, 2002) respectively.

The results showed that ARSF1 could release sulfate from fucoidan oligosaccharides after digestion with FcnAΔ229, Fda2 and FdlB with 0.62, 0.92 and 2.95% sulfate released respectively, suggestion a preference towards the oligosaccharides released by FdlB action. ARSF1 was also able to release sulfate from oligosaccharides released from *F. evanescens* fucoidan, although to a lesser extent. Fda2 produced oligosaccharides from *F. evanescens* was although not observed, while in contrast sulfate was released from oligosaccharides from pre-treatment with FcnAΔ229 and FdlB with 0.24 and 0.47% of the sulfate released respectively.

Discussion

We have successfully purified the recombinant arylsulfatase ARSF1 expressed in *E. coli*, which was originally identified from a marine bacterium *Pseudoalteromonas* sp. MB47 isolated from the gut of sea cucumbers. Bacterial arylsulfatases was earlier divided into two groups according to pH optimum (Kertesz 2000), with one group showing optimal activity at pH values of 6.5–7.1 like that of the arylsulfatase purified from *Salmonella typhimurium* (Henderson and Milazzo, 1979), and the group at higher pH values of 8.3–9.0, such as the arylsulfatase from *Pseudomonas testosterone* (Tazuke *et al.*, 1998), and *Streptomyces griseorubiginosus* S980-14 (Ueki *et al.*, 1995) and the polysaccharide specific arylsulfatase Atsa/PAO from *P. aeruginosa*, with an pH optimum of 8.9 (Beil *et al.*, 1995). The optimal pH of ARSF1 was determined to be 6.5, which would imply that it belongs to group one. The pH optimum of ARSF1 is similar to the arylsulfatase from *Flavobacterium heparium* (Henderson and Milazzo, 1979) and also the optimal pH for the arylsulfatase from *T. maritima* was determined to be 7 (Lee *et al.*, 2013).

ARSF1 was found to exhibit high thermostability, which was surprising, since it has been isolated from a mesophilic marine bacterium (Cao *et al.*, 2018). Our results showed that the enzyme was highly active at a broad range of temperatures from 30°C to 75°C, with a maximum activity at 68°C. To our knowledge, there are only two thermostable bacterial arylsulfatases

described in literature to date, the arylsulfatase from *T. maritima* (NP_229503) (Lee *et al.*, 2013) and the arylsulfatase Atsa/PAO from *P. aeruginosa* (Beil *et al.*, 1995). Atsa/PAO from *P. aeruginosa* was shown to have an optimal temperature of 57°C (Beil *et al.*, 1995), while the arylsulfatase from *T. maritima*, which is active on agar, was found to have an optimal temperature of 80°C (Lee *et al.* 2013). This suggests that ARSF1 is not the first thermophilic arylsulfatase working on marine polysaccharides and which has been isolated from a marine bacterium. In addition, the arylsulfatase Ary423 (AKL72071.1) from the deep-sea bacterium *Flammeovirga pacifica*, was found to have the optimal activity at 40°C but had a wide temperature range from 30° to 70°C (Gao *et al.*, 2015). Furthermore, recombinant Ary423 retained more than 70% of its maximum activity after 12 h of incubation at 50°C and 40% at 60°C, exhibiting thermostability at high temperatures (Gao *et al.*, 2015). ARSF1 retained more than 60% of its maximum activity after 12h of pre-incubation at 68°C, which implied that ARSF1 is thermostable at higher temperatures, than what was even tested for Ary423 (Gao *et al.*, 2015), indicating that ARSF1 is superior to Ary423 at higher temperatures.

Only very few reports on fucoidan sulfatases have been published to date and none where the sequences have been determined. One sulfatase isolated from the marine bacteria *Vibrio* sp. N-5 was found to remove sulfate from sulfated fucose and short fuco-oligosaccharides formed after fucoidanase catalyzed degradation, while the activity towards fucoidan polysaccharides was very limited (Furukawa *et al.*, 1992). A second fucoidan sulfatase was found in a partially purified preparation from the bivalve mollusk *P. maximus*. This extract was shown to effectively desulfate p-NCP, L-fucose sulfated at C2 and fucoidan polysaccharide from *A. nodosum*. However, calculations have shown that only approximately 10% of the sulfate content was removed from the fucoidan sample (Sasaki *et al.*, 1996).

ARSF1 sulfatase did not work on fucoidan polysaccharides from *S. mcclurei* and *F. evanescens*. However, it exhibited activity towards oligosaccharides produced by FcnAΔ229 and FdIB. Presumably this sulfatase eliminates sulfate from the short fuco-oligosaccharides formed by the fucoidanases. Although oligosaccharides are produced by these fucoidanases, a large amount of fucoidan will not be degraded to low enough molecular size and it is expected that not all the sulfate present in the fucoidan would be released, if the sulfatase is only specific for the sulfate on the fuco-oligosaccharides. Furthermore, since the fucose residues in the fucoidan is differentially sulfated at C2, C4 or C3, dependent on the fucoidan substrate, the sulfatase would likely be specific for only one of these sulfated positions, hence not all sulfate is expected to be released even from the fuco-oligosaccharides. We observed a release of sulfate of maximum 2.95% by the action of ARSF1 after degradation with the FdIB fucoidanase on the *S. mcclurei* fucoidan. The available amounts of sulfate on oligo-saccharides on the right position is probably low, which leads us to speculate that ARSF1 function is only part of the machinery necessary for the desulfation of fucoidan for utilization as energy source by the bacterium.

The results in our study are the first amino acid sequence of a sulfatase that cleaves sulfate from fucoidan. Moreover, our study presents a novel arylsulfatase with the promising advantage of thermostability and wide range of operating temperatures, which may be attractive for application in fucoidan oligosaccharide production.

Experimental procedures

Identification of sulfatases in the *Pseudoalteromonas* sp. genome

Protein-coding features in *Pseudoaltermonas* sp. MB47 genome were predicted with Prodigal (used as part of the prokka annotation pipeline) and subjected to annotation using Interproscan pipeline (v5.27-66.0), using all available databases and analyses. All signatures corresponding to sulfatase families and domains have been retrieved from InterPro database and used for extraction of sulfatase protein sequences.

Phylogenetic tree

Sequences were used that have previously been characterized to act on polysaccharides and sequences used for the 3D modelling and the sequence with the highest identity to ARSF1, an *E. coli* sequence. Phylogenetic analysis was performed as previously described (Mikkelsen *et al.*, 2014).

Substrates

P-nitro catechol phenyl dipotassium sulfate salt was purchased from Sigma. Crude fucoidans from *Sargassum mcclurei*, *Fucus evanescens* were extracted as described by Zvyagintseva *et al.* (1999). Fucoidan from *S. mcclurei* was purified further by ion-exchange chromatography (Thinh *et al.*, 2013). Fucoidans from *F. evanescens* was purified as described by Kusaykin *et al.* (2006).

Cloning, expression and purification of ARSF1

The crude extract of ARSF1 was applied to a 5ml Ni²⁺ Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden) which was equilibrated with binding buffer 20mM Tris-HCl buffer, 100mM NaCl, 20mM imidazole, 10mM CaCl₂, pH 7.5. The resin was washed 3 times with the washing buffer (the same binding buffer) and proteins were eluted by elution buffer with 20mM Tris-HCl buffer, 100mM NaCl, 10mM CaCl₂, 100mM to 500mM imidazole, pH 7.5. The eluted fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The protein was desalted and imidazole was removed using a PD10 column. The protein was diluted in 40mM Tris-HCl pH 6.5 contained of 10mM CaCl₂ to a final concentration of 1mg/ml. Protein content was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as standard.

Enzymes and gene constructs

The construct containing the gene encoding sulfatase was designed to harbour an N-terminal 6xhistidine tag. The synthetic codon-optimized genes (optimized for *E. coli* expression), all devoid of their original signal peptide, were synthesized by GenScript (Piscataway, NJ, USA) and delivered as inserted either into the pET-45b(+) vector between the KpnI and PacI restriction sites.

The *Escherichia coli* strain DH5α (Invitrogen® Life Technologies, Thermo Fisher Scientific, MA, USA), was used as plasmid propagation host. BL21 (DE3), BL21 (DE3) PlyS and C41 (DE3) (also from Invitrogen® Life Technologies) were used as expression hosts for sulfatase. Protein expression was done as described below.

The constructs containing the genes encoding the sulfatases were designed to harbour an N-terminal 6xhistidine tag. The synthetic codon-optimized genes (optimized for *E. coli* expression), all devoid of their original signal peptide, were

synthesized by GenScript (Piscataway, NJ, USA) and delivered as inserted into the pET-45b(+) vector between the KpnI and PacI restriction sites.

Production of recombinant ARSF1

Expression of sulfatases was performed in *E. coli* (BL21 (DE3) harbouring the Pch2 (pGro7) plasmid. Overnight cultures grown at 37°C with agitation (180 rpm) in lysogeny broth (LB) medium containing 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol were used to inoculate 500ml LB containing 100µg ml⁻¹ampicillin, 34µg ml⁻¹chloramphenicol and 0.05% arabinose. The inoculated LB was incubated at 37°C with 180 rpm shaking until OD₆₀₀ = 0.6-0.8. Enzyme expression was induced with 1mM IPTG for 20 hours at 20°C and 180 rpm. Cells were harvested by centrifugation at 5000xg for 20min and 4°C and the pellet was re-suspended in binding buffer (20 mM Tris-HCl buffer, 100 mM NaCl, 20 mM imidazole, pH 7.5) before being disrupted by UP 400S Ultrasonic processor with 0.5 cycle and 100% amplitude. Cell debris was pelleted by centrifugation (20,000×g, 20 min at 4°C). The supernatant obtained by centrifugation was then filtered through a 0.45µm filter (crude enzymes).

The crude extract of ARSF1 was applied to a 5ml Ni²⁺ Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden) which was equilibrated with binding buffer 20mM Tris-HCl buffer, 100 mM NaCl, 20 mM imidazole, 10mM CaCl₂, pH 7.5. The resin was washed 3 times with the washing buffer (the same binding buffer) and proteins were eluted by elution buffer with 20 mM Tris-HCl buffer, 100 mM NaCl, 10mM CaCl₂, 100 mM to 500mM imidazole, pH 7.5. The eluted fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described in the Chapter 2. The protein then was removed desalt and imidazole by PD10 desalting column. The protein then immediately was diluted in 40mM Tris-HCl pH 6.5 contained of 10mM CaCl₂ with the final concentration 1mg/ml to avoid the precipitation. Protein content was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as standard.

ARSF1 sulfatase was purified by 5ml Ni²⁺ Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden). The protein then was desalted and imidazole was removed using a PD10 desalting column.

SDS-PAGE

The homogeneity and molecular weight of the recombinantly expressed proteins were estimated by (SDS–PAGE) electrophoresis according to the Laemmli protocol (Laemmli, 1970). Electrophoresis was performed in 12% acrylamide gels with the addition of 4xlaemmli loading-buffer, to 40µg of crude protein and 5µg purified protein and 5mM DTT. The analysis of total intracellular proteins was conducted by using the biomass from 300µl culture with 100µl of 4xLaemmli loading-buffer, 10µl of samples were loaded on the 12% acrylamide gels. The Protein Plus molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA) with molecular weights of 10–250 kDa was used as standard.

Western blot analysis of proteins

Total intracellular protein, crude enzymes (40µg) and pure enzymes (5µg) were separated by 12% acrylamide gels with the addition of 4xlaemmli loading-buffer. Separated proteins were transferred onto a PVDF blotting membrane (GE Healthcare

No. 1060022) and blotted in Tris-glycine pH 8.3 running buffer at 100V for 45 min, after activation of the membrane in 96% ethanol for around 10 sec. The membrane was blocked with 2% skim milk in 0.01M TBS (Tris-based sodium chloride pH 7.6) buffer containing 0.1% Tween 20 (TBS_T buffer) for 60 min. The membrane was then incubated in TBS_T buffer with monoclonal anti-polyhistidine peroxidase conjugated antibody (Sigma-Aldrich, Steinheim, Germany) at 1:10.000 dilutions in a total volume of 30ml for 1 hour. The membrane was washed in TBS_T buffer 3x10 min and TBS with 0.1% Tween20 for 20 mins. The bound antibodies were detected by horse radish peroxidase using the AEC Kit (Sigma-Aldrich, Steinheim, Germany) according to manufacturer's protocol.

Enzyme Activity Assays

X-SO₄ plate screening

The crude cell lysates containing the recombinant sulfatases were tested on agar plates containing 20mM Tris-HCl, 125mM NaCl, pH 6.5 and 100µg/ml X-SO₄ substrate (Carbonsynth, United Kingdom, Compton).

pNCS and pNPS assays

The sulfatase activity during protein purification was routinely assessed by incubating the enzyme samples in 100 mL reaction mix, the final concentrations are 20mM Tris-HCl, 2.5 mM pNCS, 125mM NaCl and 0.5mg/ml of sulfatase for 1 hour (standard condition). The reaction was stopped by the addition 500µl of a 1M NaOH solution followed by measuring the UV absorbency at 515 nm. One unit of the sulfatase activity was defined as the amount of the enzyme that was capable of hydrolyzing 1 µmole of pNCS sulfate per minute.

The influence of metal ions on the enzyme activity was determined after ARSF1 sulfatase was pre-incubated with 0-10mM CaCl₂, MgCl₂, MnCl₂, NiCl₂, CuCl₂, ZnCl₂, and FeCl₂ for 5 min, at pH 7.5 1 hour of incubation at 37°C with 2.5mM pNCS.

The effect of pH on sulfatase activity was assessed following two overlapping pH buffer systems ranging from 5.0 to 8.0 (200mM acetate buffer pH 5.0 and 200mM Tris-HCl buffer at pH 6.5 and 8.0). The temperature dependence of ARSF1 activity was monitored by performing incubations with pNCS at 30, 45, 60, 60 and 75°C C (at pH 6.5). The enzymatic reaction contained 10mM CaCl₂ and 125mM NaCl.

The thermostability of recombinant ARSF1 was evaluated by measuring the residual enzyme activity after pre-incubation the enzyme in 40mM Tris-HCl, 10mM CaCl₂, and pH 6.5 at 68°C for various time durations (1h, 2h, 3h, 4h, 5h, 6h, and 12h). Pre-incubation of ARSF1 was performed in the absence of substrate.

Kinetics experiments were performed with different pNCS concentration (0.5, 2.5 and 4mM) at pH 6.5, 68°C. The enzyme activity was monitored by the amount of the enzyme that was capable of hydrolysing 1 µmole of pNCS for 1 minute. The K_m and V_{max} of sulfatase was calculated according by Michaelis-Menten.

Desulfation of fucoidans was performed after fucoidan hydrolysis catalysed by different fucoidanases in reaction mixtures containing 0.5µg/µl fucoidanase (FcnA2t and FdIB) solution in 20mM Tris-HCl buffer pH 7.4, 250mM NaCl and 10mM CaCl₂ and 1% weight/volume fucoidans from *S. mcclurei* or *F. evanescens* at 35°C for 24h. The sulfatase ARSF1 (1mg/ml)

was afterwards added to the reaction with 100mM Tris-HCl pH6.5 doubling the volume resulting in the following final concentrations: 125mM NaCl, 10mM CaCl₂, 60mM Tris-HCl pH 6.5, 0.5% fucoidan.

Sulfate detection by ion-chromatography

The sulfate contents were analysed by high performance anion exchange chromatography (HPAEC) using a Dionex ICS-5000 chromatography system equipped with a GP40 gradient pump and an ED40 electrochemical detector. The ions were separated on an HC-AS11 anion-exchange column (4 x 250 mm; Dionex) with accompanying HC-AG11 guard column (4 x 50 mm; Dionex). Elution was performed with 35mM NaOH using an isocratic flow rate of 1.5 mL/min. Background signal and noise originating from the eluent is reduced using an anion self-regenerating suppressor (AERS-500, Dionex) with a current of 180 mA. Sulfate concentration is deduced from the signal intensity and calculated from a standard sulfate calibration curve using Na₂SO₄.

3D homology modelling

The 3D structure homology model of ARSF1 was prepared by YASARA 16.9.23 (YASARA Biosciences GmbH, Vienna, Austria)(Krieger and Vriend 2014) using the built-in homology modelling function. The following PDB-structures were automatically identified as templates for the modelling: 4FDI, 1E2S, 1N2K, 1E1Z, 1E3C, 1E33, 4UPI, 5G2V, 3ED4. After preparing five models for each template YASARA combined the best parts of each model based on a quality ranking in a hybrid model. In order to prepare the model for docking the active site residue formyl glycine (FGly) was created by substitution of the present Cys111 with the respective side chain. The correct positioning of the FGly side chain was identified by comparison with the PDB structure 4FDI. This model containing the correct FGly was further refined using the `md_refine` macro as it was supplied with the YASARA package. The snapshot with the lowest energy after refinement was considered as the final model and its quality was checked by QMEAN4 (Benkert *et al.*, 2008). To enable docking of small molecules into the model structure it was equilibrated by molecular dynamics (MD) simulation over 20 ns using YASARA. First, the program was used to clean the model structure and optimize the hydrogen bonding network. Afterwards, a cubic simulation cell extending 10 Å around all atoms was created and the AMBER15IPQ force field (Debiec *et al.*, 2016) was chosen. A cell neutralization and pK_a prediction experiment at pH 6.5 was carried out to neutralize the simulation cell and assign correct protonation states of the amino acid side-chains. The resulting scene was saved as solvent system for the MD simulation, which was done using the YASARA macro `md_run` with the pressure control mode “Manometer”, pH 6.5, 0.1% CaCl₂ and 341 K over a time of 20 ns. The final simulation snapshot was used for further docking experiments. Therefore, the protein dimer was split into monomers in YASARA and both were submitted to docking experiments using the YASARA macro `dock_run` utilizing AutoDock Vina (Trott *et al.*, 2010) with 300 docking runs. pNCS, pNPS, 2-SFuc, 4-SFuc and 2,4-SFuc were used as ligands. The resulting docked structures were compared to available crystal structures with the respective co-crystallized ligands (1E2S for pNCS and pNPS, 5G2V for the sulfated fucoses) by alignment to the respective structure using the MUSTANG method (Konagurthu *et al.*, 2006) in YASARA. All models and substrate docked structures were visualized in PyMOL (ThePyMOL Molecular Graphics System, Version 1.1 Schrödinger, Cambridge, MA, USA).

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Figure legends

Figure 1 Phylogenetic tree of selected S1 family sulfatases. **Green:** *Pseudoalteromonas* sequences (sea cucumber), **Pink:** Sequences with 3D structures used to generate the 3D modelling of ARSF1, **Red:** Ary432 from *Flammeovirga pacifica* –, **Blue:** ARS from *Pseudomonas aeruginosa*, *Thermotoga maritima* NP_229503. Accession numbers are included in the tree. The phylogeny was constructed using Neighbor-joining algorithms, with gaps removed.

Figure 2 Purified recombinantly expressed ARSF1 sulfatase. A) SDS-PAGE B) Western blot of (St) protein plus molecular weight marker, A) unstained, B) prestained. The expected molecular weight of the recombinant sulfatase ARSF1 was 57 kDa.

Figure 3 3D homology model of ARSF1. The 3D structure shows a β -sandwich fold. The structure is predicted as a homodimer and a calcium ion (green sphere) is predicted in each active site. The calcium coordinating amino acid residues incl. the active site formyl glycine (yellow sticks) are shown as grey sticks.

Figure 4 Docking of sulfated β -fucose into the modeled active site of ARSF1. As small model substrates fucose-2-sulfate (A), fucose-4-sulfate (B) and fucose-2, 4-disulfate (C) were docked into the homology model of ARSF1. The predicted calcium ion is shown as green sphere, the calcium coordinating residues incl. the active site formyl glycine (yellow sticks) are shown as grey sticks. The docked model substrates are shown as cyan sticks and residues in substrate binding by polar contacts (yellow dotted lines) are shown as magenta sticks.

Figure 5 Docking of aromatic substrates into the modeled active site of ARSF1. As model substrates p-nitrophenyl sulfate (A) and 4-nitrocatechol sulfate (B) were docked into the homology model of ARSF1. The predicted calcium ion is shown as green sphere, the calcium coordinating residues incl. the active site formyl glycine (yellow sticks) are shown as grey sticks. The docked model substrates are shown as cyan sticks and residues in substrate binding by polar contacts (yellow dotted lines) are shown as magenta sticks.

Figure 6 Surface response curve as a function of temperature and pH on ARSF1 sulfatase activity. The incubation mixture contained 10mM Ca^{2+} . The curve in plot is isorates (in all the points of the value of activity is the same). The color varies from blue (low sulfatase activity) to red (high sulfatase activity). The optimal pH of the sulfatase was found to be 6.5 and the optimal temperature 68°C.

Figure 7 Thermostability of the recombinant ARSF1 sulfatase. The residual activity of ARSF1 was determined by pre-incubation of ARSF1 at 68°C for different time durations. ARSF1 retained approximately 60% of its maximum activity after 12h of incubation at 68°C, exhibiting good thermostability at high temperatures.

Figure 8 Sulfate release from fucoidan oligosaccharides by ARSF1. Fucoidan from *Sargassum mcclurei*, the products of the enzymatic reaction of fucoidanase FcnA Δ 229, Fda2, FdlA, FdlB was used as the substrates ARSF1 sulfatase. Dextran sulfate with different size (4, 14 and 40 kDa) was also used for monitor sulfatase activity.

Tables

Table 1 Effect of divalent cations on ARSF1 activity

Divalent cations	Relative activity (%)			
	0mM	2mM	5mM	10mM
Ca ²⁺	100	258	452	608
Mg ²⁺	100	110	124	127
Mn ²⁺	100	131	nd	nd
Ni ²⁺	100	16	15	nd
Zn ²⁺	100	6	nd	nd
Cu ²⁺	100	35	nd	nd
Fe ²⁺	100	8	nd	nd

nd: not determined.

Table 2 Activity of ARSF1 on different fucoidan substrates

Substrates	SO ₄ released (%)
Fucoidan from <i>S. mcclurei</i>	0.00
<i>S. mcclurei</i> – FcnAΔ229	0.62
<i>S. mcclurei</i> – Fda2	0.92
<i>S. mcclurei</i> – FdlB	2.95
Fucoidan from <i>F. evanescens</i>	0.00
<i>F. evanescens</i> - FcnAΔ229	0.24
<i>S. mcclurei</i> – Fda2	0
<i>S. mcclurei</i> – FdlB	0.47

Figure 1

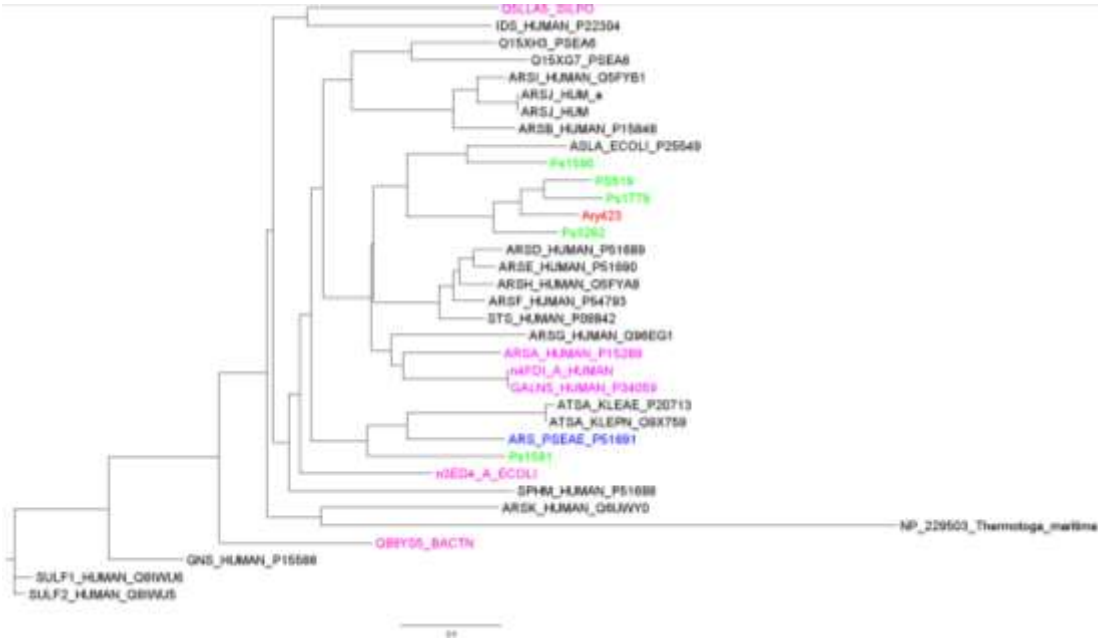
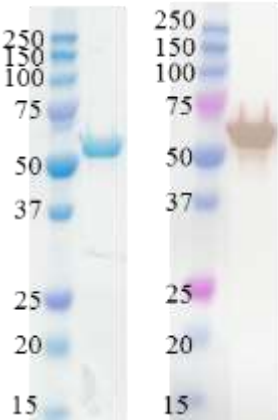


Figure 2

A



B



Figure 3

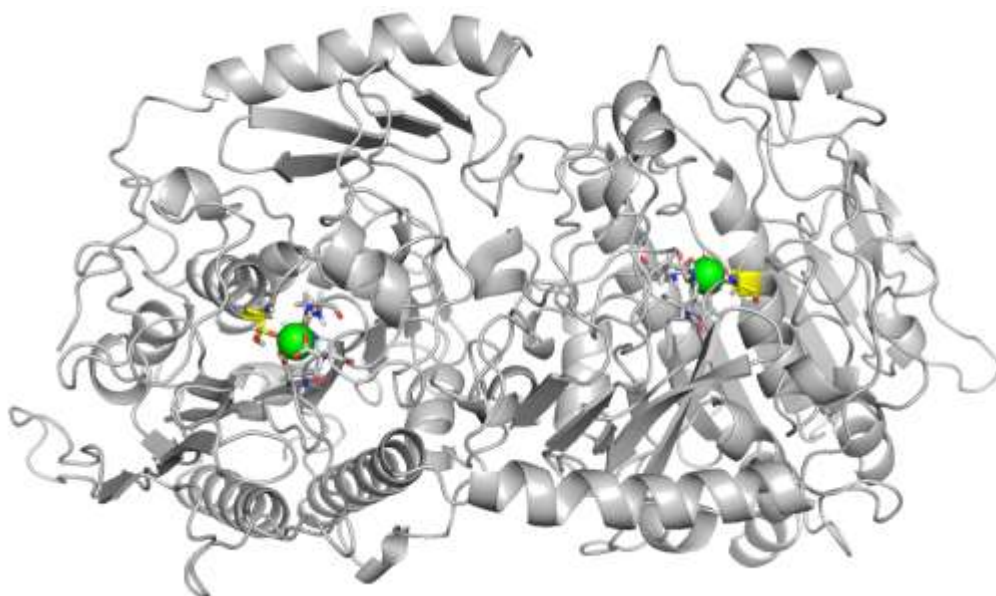


Figure 4

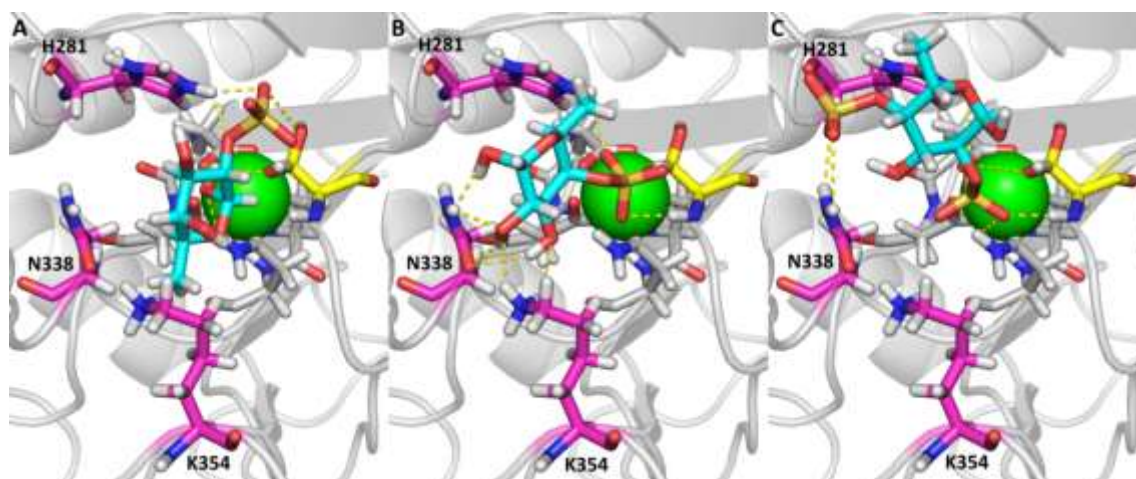


Figure 5

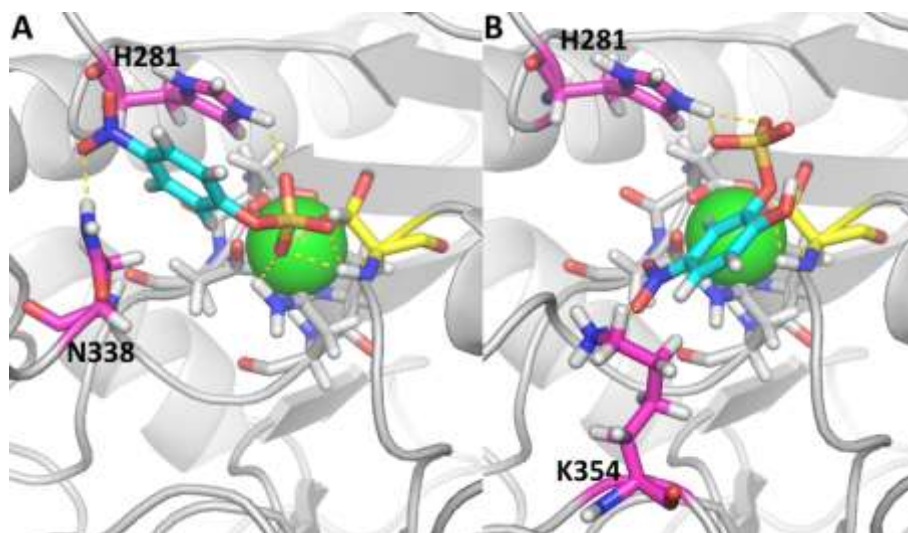


Figure 6

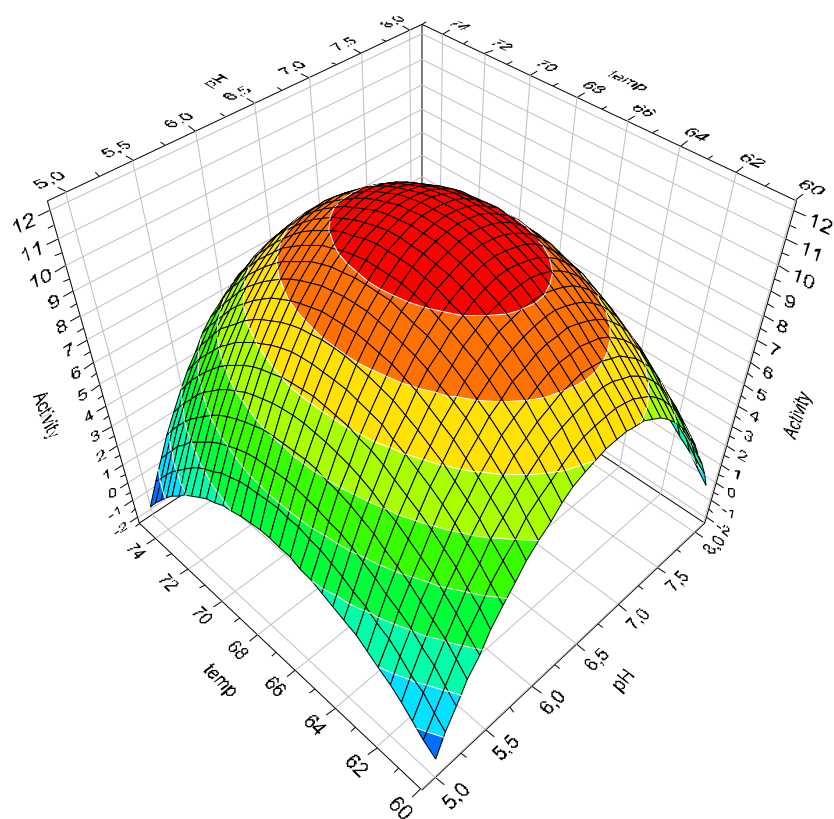


Figure 7

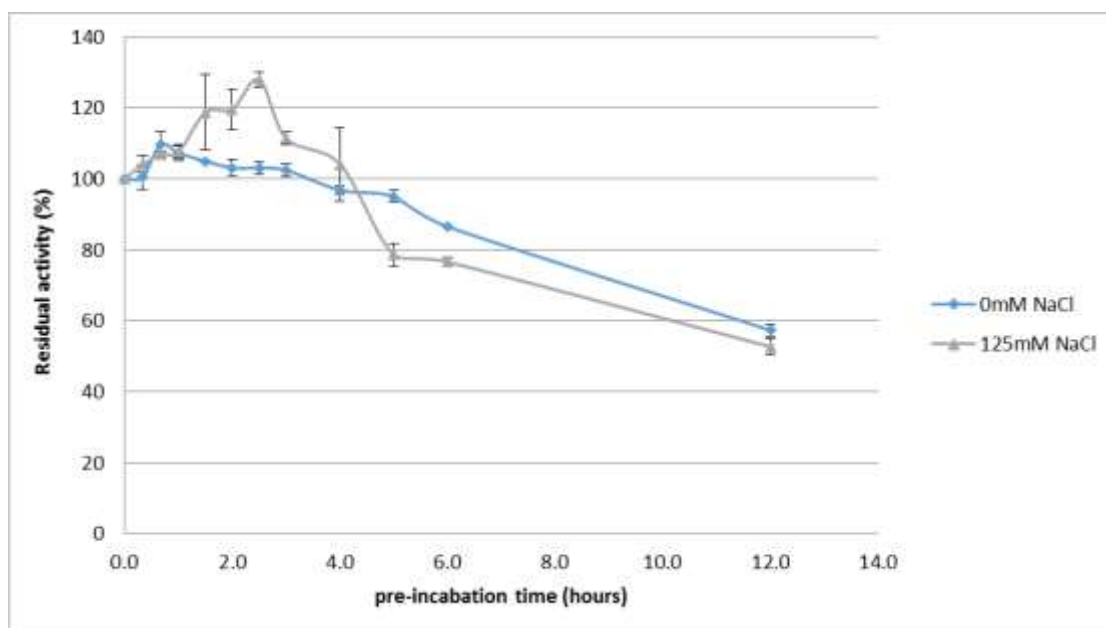
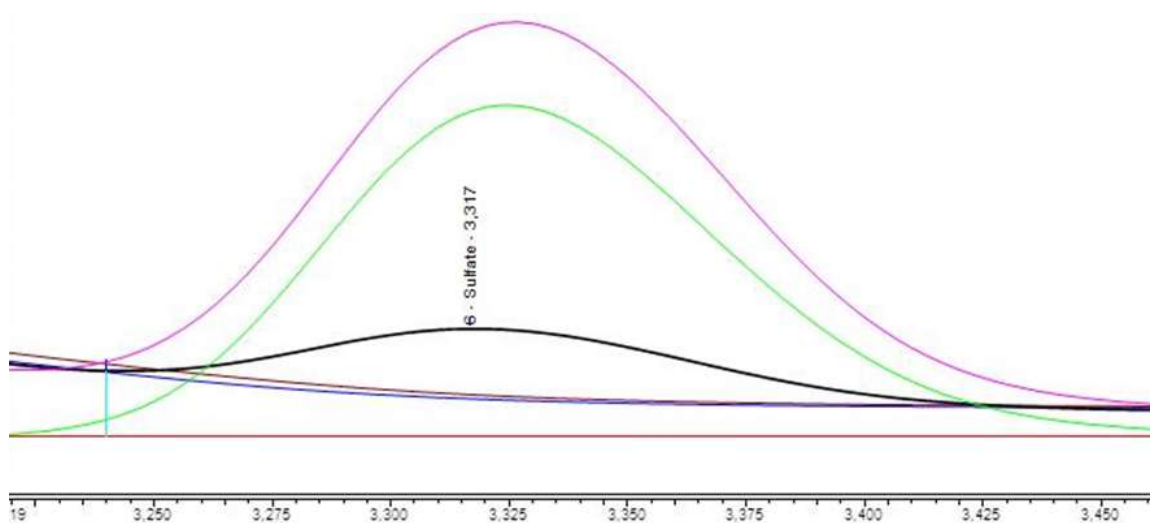


Figure 8



Supplementary Figure legends

Figure S1 Sulfatase activity on *E.coli* cells and crude extracts. A) *E.coli* cells harbouring the sulfatases streaked on X-SO₄ containing agar plates: MH07) ARSF1, MH08) ASUL3, MH09) AER35705, MH10) Ps3148 and MH11) AtsA. AtsA cells turned blue after 24h at 37°C, indicating sulfatase activity. B) Crude extracts from *E. coli* cells 1) ARSUL1, 2) ARSUL2, 3) ARSF1, 4) ARSUL3, 5) Ps3148, 6) ARSUL4, 7) ARS, 8) Ary432, and 9) AER35705. ARS and ARSF1 showed activity by formation of blue color where the enzyme had been added, due to release of sulfate.

Figure S2 The effect of NaCl on ARSF1 sulfatase activity. Desulfation of pNCS by the sulfatase showed that the maximum activity is reached within 30min in the absence of NaCl, while at 62.5mM and 125mM NaCl the maximum is reached in approximately 40 or 50 min respectively.

Figure S3 Thermal stability of the recombinant ARSF1. A) Reaction mixture without NaCl, B) Reaction mixture with 125mM NaCl. The residual activity of ARSF1 was determined by pre-incubation of ARSF1, in the absence of substrate, at 68°C for different time duration. A semi logarithmic linear plot was obtained for time vs. the Ln of the specific activity (SP Activity, U/mg). The first order rate constant of the thermal denaturation (kd) was obtained from the slope.

Figure S4 Michaelis-Menten kinetics of ARSF1.

Figure S5 Predicted protein sequences of the proposed sulfatase from *Pseudoalteromonas* sp.

Figure S6 Predicted domains in the sulfatase from *Pseudoalteromonas* sp. domains were predicted using ProSitePatterns, Pfam and SulfAtlas

Supplementary Tables

Table S1 Sulfatase, features, molecular weight

Enzyme name		Organism	Features ^a	bp	Length (aa) ^b	Expected molecular weight (kDa)
1	ARSUL1	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	1482	494	57kDa
2	ARSUL2	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	2271	757	90kDa
3	ARSF1	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	1521	507	57kDa
4	ARSUL3	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	1440	480	55kDa
5	Ps3148	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	3117	1039	116kDa
6	ARSUL4	<i>Pseudoalteromonas sp.</i>	His ₆ (N-term)	1467	489	56kDa
7	ARSP51691	<i>Pseudomonas aeruginosa PAO1</i>	His ₆ (N-term)	1611	537	61kDa
8	Ary432 AKL72071	<i>Flammeovirga pacifica</i>	His ₆ (N-term)			56kDa
9	AER35705	<i>Pseudoalteromonas carrageenovora</i>	His ₆ (N-term)			117kDa

^a Wild type signal peptide had been removed for codon-optimized synthesized construct.

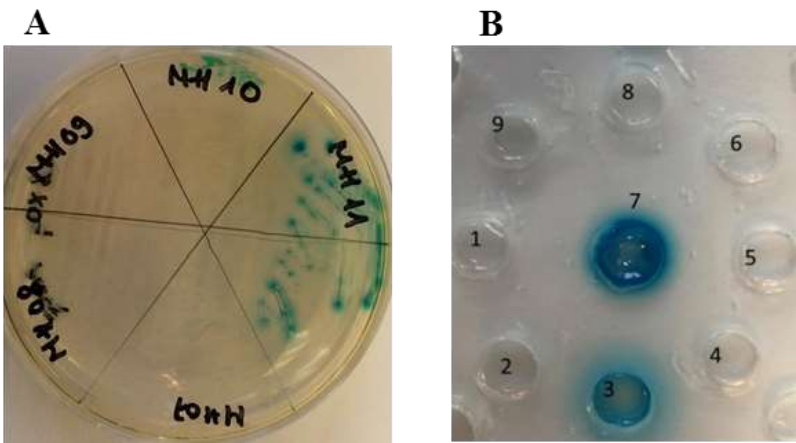
^b Including the his-tags

Table S2. Predicted protein coding features with sulfatase signatures detected by InterProScan.

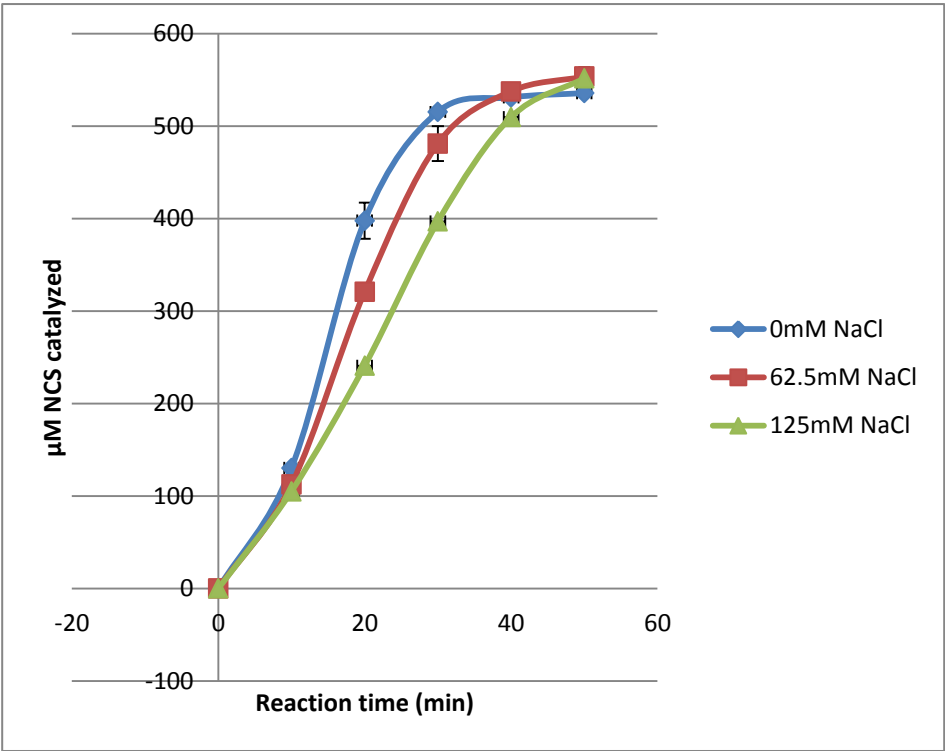
Protein Accession	Sequence Length	Analysis	Signature Accession	Signature Description	Start location	Stop location	Score	InterPro annotation accession	InterPro annotations	GO annotations
IMPJCBK J_00307	524	Pfam	PF00884	Sulfatase	61	399	1.2E-060	IPR000917	Sulfatase, N-terminal	GO:0008152 GO:0008484
IMPJCBK J_00307	524	ProSitePatterns	PS00149	Sulfatase signature 2.	158	168	-	IPR024607	Sulfatase, conserved site	
IMPJCBK J_00303	780	ProSitePatterns	PS00523	Sulfatase	109	121	-	IPR024607	Sulfatase, conserved site	

				signature 1.							
IMPJCBK J_00303	780	Pfam	PF00884	Sulfatase	65	479	2E- 084	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84
IMPJCBK J_00492	498	Pfam	PF00884	Sulfatase	23	352	3E- 062	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84
IMPJCBK J_02757	514	Pfam	PF00884	Sulfatase	25	371	8.9E -068	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84
IMPJCBK J_02557	508	Pfam	PF00884	Sulfatase	24	356	7E- 063	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84
IMPJCBK J_03684	541	Pfam	PF00884	Sulfatase	237	521	7.2E -045	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84
IMPJCBK J_03683	647	Pfam	PF00884	Sulfatase	279	552	1.1E -035	IPR00091 7	Sulfatase, terminal	N-	GO:000815 2 GO:00084 84

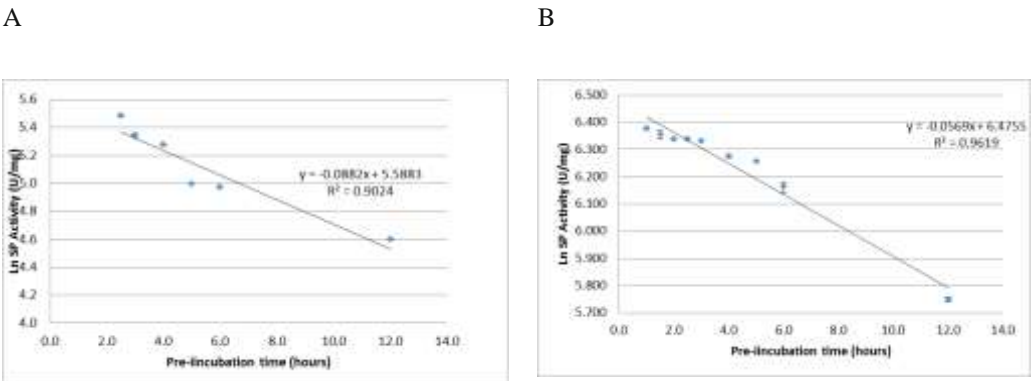
Supplementary Figure 1



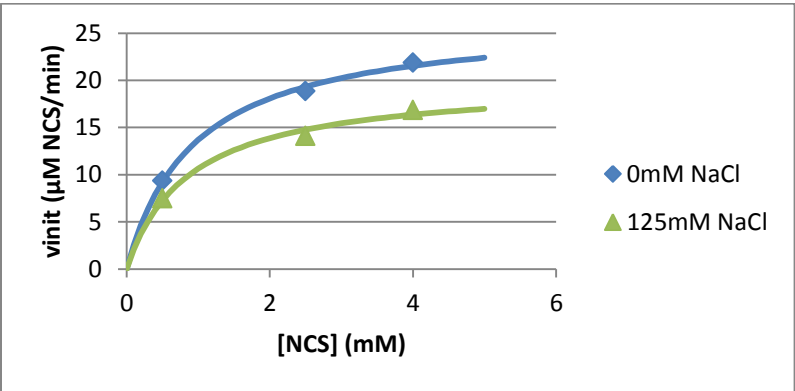
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



NaCl (mM)	K_M (mM)	V_{\max} ($\mu\text{M}_{\text{pNCP}}/\text{min}$)
0	0.95 ± 0.15 (100%)	26.6 ± 1.2 (100%)
125	0.88 ± 0.25 (92%)	19.9 ± 1.6 (75%)

Supplementary Figure 5

>IMPJCBKJ_00303 Arylsulfatase ARSUL2

MNRLKRVGSIIISLLLLSGFSQVNASEIDRSVLPAAPEFQGKIGKTFENSKQDYPQPIKAPKGAPNVVVILLDDFGFG
HPSTFGGPIPTPSIDKLANEGLKFNRFHHTAICSPTRAALLTGRNHHQVAMGTITELSTGYPGYHSVWPDETASIAQI
LKENGYNTAAWGKWHNTPDWETSPIGPFHDWPTGLGFEYFYGFQGGETSQWEPQLFRNTTPVEPPKKPEQGYHL
TEDLVDDAIKWMGQQESIDSEKPFMYFATGATHAPLHAPQEWNDKFKGQFDQGWDKVREETLARQKSLGIAPQ
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IKEHGGRLRSQFHHVIDIAPTILDAAHIEPKVVNGVEQTPMAGVSMLYSLNDKDAK GKRTTQYFETGGHRAIYHDG
WVAASFHGVPAWALNGSVGFDNDKWELYNIEEDFSEAVDLSAKYPDKLRTLISIFDQEAKKFNVYPLDDRFVERGT
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>IMPJCBKJ_00307 Arylsulfatase ARSF1

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PGVYGGGAAIGAPTPNIDKLANEGLRLTSMYSQPTCTSSRAALTTGRLPVRSGLV RPILTGDKVTQNPWEKEVSQG
KLLSKVGYKTALIGKWHVGEAEGMLPHEVGFDYFYGLPSVQSDYTQFLVERQYADMMTNKELYTKASQLRPEG
LIKGRKGGKREVAYPINSIEDISMIDQVLRDES VKFINQAVDEGKPFYLIHSFSKIHNDNYPAPKYKGASPAAMPVR
DAMVEVDDITGELVALLKEKGQLENTLIIFTSDNGPNEDTWPDSGYSPWRGGKGTWEGGVRIPGIAYWKG MISA
GQVNNGLMDLTDIYMTSLRLGGVIDELPSNMYFDGIDQTAFLADNGKSRRQVVYMW SREDFTALRWLDYKIH F
KVFN TAVPRRNIDASFLLDIGTAPWVFNLNMDPKEMASTGHQYFEWGMPQATKFMKAHIATMKKYPNTDIGLGL

>IMPJCBKJ_00492 Arylsulfatase ARSUL3

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NVSEEPEQADYPKDPKFVKKFGPRGVIESYANGKITDTGPLTRKRMETFDEEV LERSLNFMERAVKAKKPFFI WHA
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>IMPJCBKJ_02557 Arylsulfatase ARSUL4

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KQNKPFVWHNTTRMHYQTNLNKAYDGKTGFGLYADGMTEMDDDDVGELLNLLDELKVADNTIVMFSTDNGAA
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MDPFERFIDARGYDEWAENRSWILGPAGNQISEFVKTFKAFPPSQESMSVQVGSVAEMISQQALNR

>IMPJCBKJ_02757 Arylsulfatase ARSUL1

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TAGRAALITGQYPIRSGMTTVGRPGAELGLKAGSYTLAEVLKEQGYATGQFGKNHLDGRNEHLPTVHGFDEFFGN
LYHLNTQEEFEQRDYPTDPEFFKKYGTGRVLTHTYATNVDDKTVDPRFGKVKGKQKIKDTGQLSRERMKTVDKEFM
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NIRMDPFESFDGLTDRSDIVQAKQWLNEPVQHLLGEHIKSLSEYPPLQKAASFDFSQLEQLMSGKQ

>IMPJCBKJ_03683 Lipoteichoic acid synthase 1

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YRYINTAFSKQQNAKTVGQVVILVTLLVCLISARGTVSHRPLNPALVYFSSDSLNSLALNSTYSVAFAIKNMGN
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>IMPJCBKJ_03684 Phosphoethanolamine transferase EptA

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TFVKPVLLTSIVSSLLFYATLNYGVIFDKSMLQNILETNSGEAFSYLNEQVIAFITVLGVLPAYLFSRFKIVGNFRIR
LKSFAFLNVTAFIAVAVIAVPFYKDYASVGRNNRQLTSYITPFAFYTAGYKYLRDNYFYPPPLPFKVLDKTPYLLAK
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GEKGLYLHGFYALAPKEQTHVPMLFWSNKLANTRFNQICIEQQRNHAFSHDNIFDTLLGLTHIQSTEYRPEQDIFN
TCSTQS

>Ps3148 Amidohydrolase

MKKMLHTALAVSISLALTGQVAAAEQSNDDKWQVDAPKGQFLDAKISVEQGTWMNVDISPDGKTVVFDLLGDI
YTMPMSGGTATQLTSDIAWQMQRFPSPDGKHIAFTSDQGGGDNIWMDLNGENQKAVTNETFRLLNSPAWSPDG
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KLYFEKI*

Novel enzyme actions for sulfated galactofucan depolymerisation and a new engineering strategy for molecular stabilization of fucoidan degrading enzymes

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Abstract

Five recombinant microbial fucoidan degrading enzymes were evaluated on distinct fucoidan structures of varying backbone constitution from brown macroalgae: *Sargassum mcclurei*, *Fucus evanescens*, *Fucus vesiculosus*, *Turbinaria ornata*, *Saccharina cichorioides*, and *Undaria pinnatifida*. The enzymes included three endo-fucoidanases (EC 3.2.1.-, GH 107): FcnA2, Fda1, Fda2, and two unclassified endo-fucoglucuronomannan lyases: Fd1A and Fd1B. Oligosaccharide product profiles after enzymatic treatment of the fucoidan substrates were assessed by carbohydrate-polyacrylamide gel electrophoresis (C-PAGE) and size exclusion chromatography. Fucoidans containing $\alpha(1\rightarrow4)$ -linked L-fucosyls were degraded. Fda2 degraded *S. cichorioides* and *U. pinnatifida* fucoidans that both solely have $\alpha(1\rightarrow3)$ -linked L-fucosyls in their backbone. A stabilized form of Fda1 could also cleave $\alpha(1\rightarrow3)$ bonds. For the first time we show that several enzymes can degrade *S. mcclurei* galactofucan-fucoidan. The recombinant FcnA2, Fda1, Fda2 were unstable to different extents. However, active and more stable enzymes were obtained by truncation of the C-terminal end (by removal of up to 47% of the protein in Fda1). The data enhance our understanding of fucoidan degrading enzymes and their substrate attack preferences and have implications for development of new enzyme-based procedures for producing fucoidan oligomers from different fucoidan substrates.

Key words: fucoidan, endo-fucoidanase, galactofucan, molecular stabilization, *Sargassum mcclurei*, *Turbinaria ornata*

Introduction

Fucoidans designate a family of sulfated, fucose-rich polysaccharides uniquely produced by brown marine macroalgae (seaweeds) and certain marine invertebrates such as sea cucumbers^{1, 2}. In general, fucoidans, or fucose-containing sulfated polysaccharides, FCSPs, consist of a backbone of α -L fucosyl residues linked together by (1 \rightarrow 3) and/or (1 \rightarrow 4)-glycoside bonds which are organized in stretches of α (1 \rightarrow 3) or of alternating α (1 \rightarrow 3)- and α (1 \rightarrow 4)-glycoside bonds depending on the macro algae origin (species; age; geographical origin; collection season) of the fucoidan³. The L-fucosyl residues may be sulfated ($-\text{SO}_3^-$) at positions C2 and/or C4 (rarely at C3). Some fucoidans may have fucose, galactose, glucuronic acid or other mono- and oligosaccharides as short branches^{1, 4, 5}. Galactofucans are the most structurally diverse group of fucoidans synthesized by brown algae, having galactose in either the backbone or as branches. The position and content of galactose residues in various galactofucans depend on the type of algae^{6, 7}.

The fucoidan from *Fucus vesiculosus*, which is available commercially, is known to be made up of a backbone of repeating disaccharide units of α (1 \rightarrow 3)- and α (1 \rightarrow 4)-linked sulfated L-fucosyl residues (C2, C2/C3, C2/C4, C4 sulfatation)^{8, 9, 10}.

The fucoidan from *Fucus evanescens* has a similar L-fucosyl backbone of alternating α (1 \rightarrow 4) and α (1 \rightarrow 3) L-fucosyls being sulfate substituted at C2. Additional sulfate occupies position 4 in some of the α (1 \rightarrow 3)-linked fucosyls, whereas a part of the remaining hydroxyl groups is randomly acetylated¹. In contrast, the bonds in the backbone of fucoidan from *Undaria pinnatifida* and from *Saccharina cichorioides* are exclusively α (1 \rightarrow 3), and the backbone *U. pinnatifida* fucoidan is moreover assumed to be rich in 2,4-disulfate substituted fucosyl residues and to contain some α (1 \rightarrow 3)-linked galactosyl residues¹¹. Some fucoidans have even more complex backbone structures as is the case for e.g. fucoidan from the brown macroalgae *Sargassum mclurei* and *Turbinaria ornata* commonly found along the Pacific Ocean coastline of Vietnam. The *S. mclurei* fucoidan is essentially a sulfated galactofucan polysaccharide having both α (1 \rightarrow 3) and α (1 \rightarrow 4) linked fucosyl residues and galactosyl- α (1 \rightarrow 4) and α (1 \rightarrow 6) linkages in the backbone. The fucosyl residues in *S. mclurei* fucoidan are moreover differentially sulfated at C2 and/or at C4 and some of the galactosyl moieties are even sulfated at C6¹². Fucoidan extracted from *T. ornata* that were collected at Nha-Trang bay, Vietnam, can also be categorized as a galactofucan. The backbone of *T. ornata* fucoidan has thus been proposed to consist of α (1 \rightarrow 3)-linked L-fucosyls with galactosyl branches (Fuc:Gal \approx 3:1) and a high sulfate content of \sim 25% with the sulfate attached mostly at C2, and to lower degree at C4, of both the fucosyl and the galactosyl residues^{13, 14}. The structural diversity of fucoidans or FCSPs is thus very high as both the sulfatation pattern and the backbone bond pattern of α (1 \rightarrow 3) and α (1 \rightarrow 4)-glycosidic bonds vary significantly depending on the fucoidan source.

The biological function of fucoidans in brown macroalgae is uncertain, but fucoidans have long been known to exert beneficial biological activities including anti-tumorigenic, immune-modulatory, anti-inflammatory, anti-coagulant and anti-thrombotic effects as demonstrated *in vitro* and *in vivo* in animal studies^{14, 15, 16}. However, the high molecular weight, irregular structure, and viscosity of fucoidans is an obstacle for providing homogeneous preparations that can be processed in a soluble form and employed for pharmaceutical purposes in sufficiently high concentrations. One approach to solving this problem is to use enzymes to depolymerize the fucoidans.

About 20 microorganisms, mainly marine bacteria, have been described that produce fucoidanases^{17, 18, 19, 20, 21}. In addition, a few fucoidanases have been found in marine mollusks^{22, 23}. In 2006 the gene encoding a fucoidanase from the marine bacterium *Mariniflexile fucanivorans* SW5T was cloned and the recombinant enzyme called FcnA. The C-terminal truncated version of FcnA called FcnA2, was previously reported to exert endo $\alpha(1\rightarrow4)$ action on fucoidan from *Pelvetia canaliculata* (a type of fucoidan encompassing both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow3)$ fucosyl-linkages in the backbone)²⁴. Furthermore, already in 2002, the genes encoding two endo-fucoidanases referred to as Fda1 and Fda2 from the marine bacterium *Alteromonas* sp. SN-1009 were sequenced and their use for degradation of sulfated fucoidan originating from the brown seaweed *Kjellmaniella crassifolia* (now called *Saccharina sculpera*) was patented²⁵. In the patent, these enzymes were reported to catalyse cleavage of $\alpha(1\rightarrow3)$ -glycosidic bonds in the *K. crassifolia* (*S. sculpera*) fucoidan²⁵. FcnA, Fda1, Fda2 all belong to the new glycoside hydrolase family GH107 in CAZy²⁶. In 2017, two endo-fucoidanases, FFA1 and FFA2, from the marine bacterium *Formosa algae* (KMM 3553^T) were characterized and also suggested to belong to GH family 107^{27, 28}. The FFA2 enzyme was proposed to be a poly[(1 \rightarrow 4)- α -L-fucoside-2-sulfate] glycans hydrolase²⁷. Already in 2003 Sakai et al. reported the finding of a new type of extracellular endo-fucoidan-lyase activity from “*Fucobacter marina*” SA-0082, or more correctly *Flavobacterium* sp. SA-0082, which acted on sulfated fucoglucurono-mannan from *K. crassifolia* (*S. sculpera*)^{29, 30}. By sequence analyses it was found that this lyase activity was apparently encoded by two separate coding regions. Recombinant expression of these two putative fucoidan degrading enzymes, referred to as FdlA and FdlB, respectively, showed that the two enzymes had about 56% amino acid sequence identity and both were claimed to act as (glucurono-) fucoidan lyases on *K. crassifolia* (*S. sculpera*) fucoidan²⁵.

The objective of this work was to compare the catalytic properties, notably the substrate degradation patterns, on different fucoidans of the three GH107 endo-fucoidanases (EC 3.2.1.-) referred to as FcnA2, Fda1, Fda2, and the two enzymes previously reported to be endo-fucoglucuronomannan-lyases, referred to as FdlA and FdlB. The distinct action of the enzymes on different fucoidan substrate structures was compared by assessing oligomer product profiles resulting after treatment with recombinantly produced enzymes on fucoidans originating from six different types of brown seaweeds: *Sargassum mclurei*, *Turbinaria ornata*, *Fucus evanescens*, *Fucus vesiculosus*, *Saccharina cichorioides*, and *Undaria pinnatifida*. We also report stabilization of the recombinantly produced enzymes by targeted gene truncation resulting in deletion of large parts of the C-terminal end of several of the enzymes.

Results and discussion

Recombinant enzyme expression

FcnA2, FdlA and FdlB all expressed well and the purified enzymes gave the expected band sizes as assessed by SDS-PAGE (Fig. 1A). Fda2 expressed with high level of degradation and it migrated slower in the SDS-PAGE gel than expected (94kDa). However, in particular for FcnA2 and Fda2 more than one band was visible in both the SDS-PAGE gel and in the western blot (Fig. 1). The congruity between these bands in the SDS-PAGE and the western blot, and presence of multiple bands in the western blot analysis, indicated spontaneous degradation rather than impurities from other proteins. This observation agrees with previously published data for recombinantly expressed FcnA2 reporting “co-elution” with other proteins, which could not be separated by anion exchange or SEC²⁴. For the Fda2 enzyme, the degradation happened during the expression in *E. coli* cells and during purification.

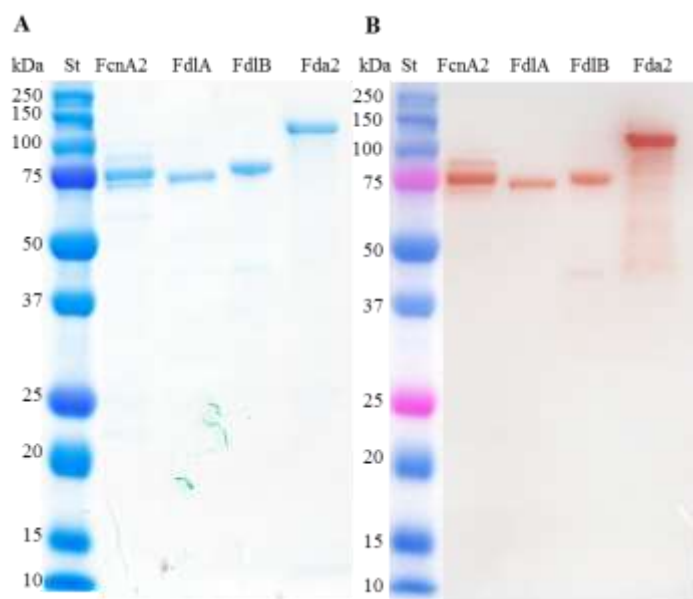
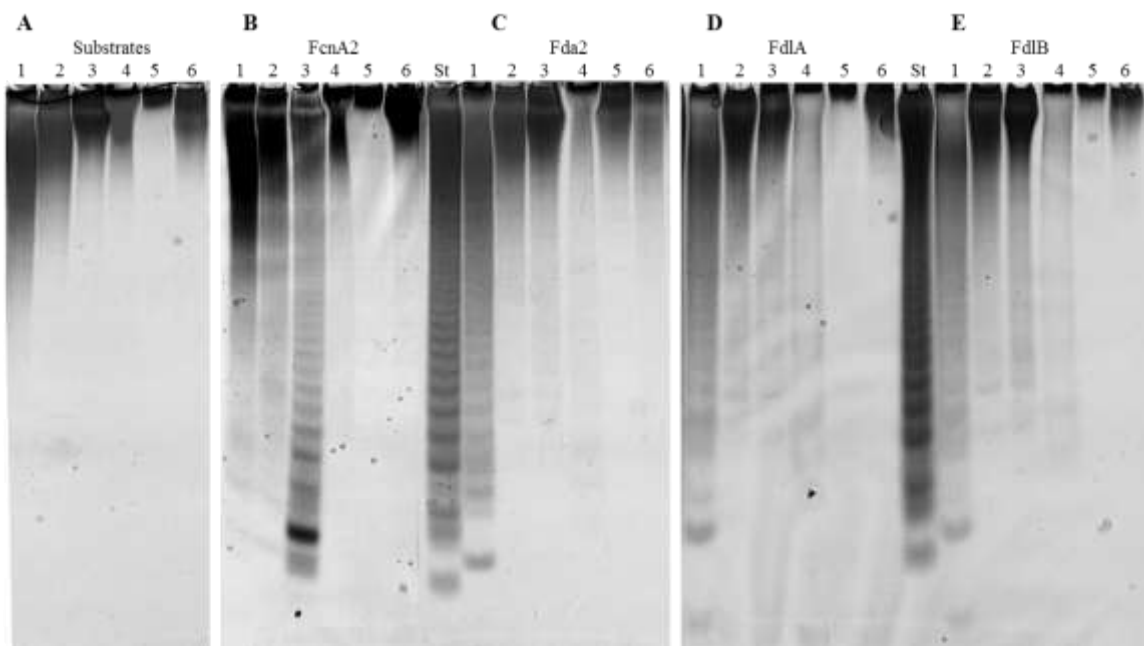


Figure 1. Purified recombinantly expressed fucoidan-modifying enzymes. A) SDS-PAGE and B) Western blot of purified FcnA2, FdlA, FdlB, and Fda2. (St) is the protein plus molecular weight marker. The expected molecular weights of the recombinant enzymes FcnA2, FdlA, FdlB, and Fda2 were 87, 75, 76, and 94 kDa, respectively. The multiple bands seen for FcnA2 and Fda2, notably in the western blot indicate partial degradation of the proteins.

The expression of recombinant Fda1 was high but the protein was retained in the cell debris after sonication (Supplementary material Fig. S1). Several culture conditions for enzyme expression (temperature, medium, IPTG concentration) were tested to obtain soluble enzyme, but without success.

Substrate specificity of the recombinant fucoidan-degrading enzymes

Treatment of six different fucoidan samples with the individual enzymes FcnA2, Fda2, FdlA, FdlB produced different C-PAGE electrophoresis patterns with the six fucoidan samples. The data indicated more profound degradation of the fucoidan substrates from *Sargassum mcclurei* (1), *Fucus vesiculosus* (2), and *Fucus evanescens* (3), than of substrates predominantly having α (1→3) glycoside bonds in their backbone structures, originating from *Turbinaria ornate* (4), *Saccharina cichorioides* (5), and *Undaria pinnatifida* (6), respectively (Fig. 2). The positive control standard (st) was the hydrolysate from enzymatic reaction of the *Formosa algae* FFA2 on *F. evanescens* fucoidan with the lowest band corresponding tetra-saccharide of (1→4) and (1→3)-linked α -L-fucosyls with each fucosyl residue sulfated at C2)²⁷ (Fig. 2). In general, the data obtained showed that all the enzymes produced differently sized sulfated oligomers in the C-PAGE chromatograms, suggesting that the different enzymes target different linkages and/or differently sulfated fucosyl residues. Furthermore results also suggest that all investigated enzymes were endo-acting. The enzymatic action left behind relatively high molecular weight fractions.



Enzymes	Substrates					
	<i>Sargassum mcclurei</i>	<i>Fucus vesiculosus</i>	<i>Fucus evanescens</i>	<i>Turbinaria ornata</i>	<i>Saccharina cichorioides</i>	<i>Undaria pinnatifida</i>
FcnA2	+	++	+++	-	-	-
Fda2	+++	+	+	+	(+)	(+)
FdlA	+++	+	+	+	-	-
FdlB	+++	+	+	+	-	-

Figure 2. C-PAGE product profiles of fucoidan degradation using purified enzymes. A) Substrate control; B), C), D) and E) enzymatic reaction of FcnA2, Fda2, FdlA, FdlB on different fucoidans respectively: 1) *Sargassum mcclurei*; 2) *Fucus vesiculosus*; 3) *Fucus evanescens*; 4) *Turbinaria ornata*; 5) *Saccharina cichorioides*; and 6) *Undaria pinnatifida*. The lowest band of the standard (St), resulting from FFA2 treatment of fucoidan from *F. evanescens*, corresponds to a tetra-saccharide of (1→4) and (1→3)-linked α -L-fucosyls with each fucosyl residue sulfated at C2²⁷. The extent of degradation is indicated with: +++ highest, ++ medium, + lowest and (+) is positive activity resulting in a high molecular smear, (-) is no activity. The reaction time was 24 hours.

FcnA2 catalyses cleavage of α (1→4) fucosyl bonds in sulfated fucoidan backbones

The recombinantly expressed FcnA2 enzyme exerted highest activity on the fucoidan from *F. evanescens*, and the degradation of this substrate was much more profound than on *F. vesiculosus*, despite the fact that both substrates have similar alternating α (1→3) and α (1→4) glycoside bonds in the backbone. The degradation of fucoidan from *F. evanescens* was in agreement with previous data reported for FcnA2 showing that FcnA2 was able to degrade fucoidan from *Pelvetia canaliculata*²⁴. Fucoidans from *F. evanescens* and *P. canaliculata* presumably have less if any C2, C4 disulfate in the “-1” position of the α (1→4)-L-fucosyl linkage compared to fucoidan from *F. vesiculosus*, which presumably contains many fucosyl residues with C2/C4 and even some with C2/C3 disulfatation. The lack of C2/C4 and C2/C3 disulfatation might be the reason for the *F. evanescens* fucoidan being more degraded than the *F. vesiculosus* (Fig. 2). Hence, FcnA2 most likely catalyse cleavage of (1→4)- α -glycosidic bond between the -1 fucosyl residues having the sulfate group occupied at C2, but not with the sulfate group occupied at both C2, C4. The differences in the degradation of the fucoidan from *F. evanescens* and *F. vesiculosus* indicate that differences in the

sulfatation pattern or in other types of substitutions on the substrate backbones may influence the action of FcnA2 on these two *Fucus* sp. derived fucoidans. The presumed presence in *F. vesiculosus* of fucosyl residues with disulfate at C2, C4 (on either the -1 or +1 position of the $\alpha(1\rightarrow4)$ glycoside bond) could thus retard the enzymatic action of FcnA2.

The smallest oligomers released from *F. evanescens* by FcnA2 were also different from those of the FFA2 treatment of fucoidan from *F. evanescens* in the standard (st) (Fig. 2). FFA2 catalyses the cleavage of $(1\rightarrow4)$ - α -glycosidic bonds in the fucoidan from *Fucus evanescens* within a structural fragment $[\rightarrow3)\text{-}\alpha\text{-L-Fucp2S-(1}\rightarrow4)\text{-}\alpha\text{-L-Fucp2S-(1}\rightarrow\text{)]}_n$ but not in a fragment $[\rightarrow3)\text{-}\alpha\text{-L-Fucp2S,4S-(1}\rightarrow4)\text{-}\alpha\text{-L-Fucp2S-(1}\rightarrow\text{)]}_n$. The difference in the oligomers released suggests that the sulfatation preferences of the FFA2 and FcnA2 may differ.

FcnA2 also catalysed degradation of the sulfated galacto-fucan fucoidan from *S. mcclurei* resulting in production of several low molecular weight bands in the C-PAGE (Fig. 2B). The partial degradation is in agreement with the enzyme attacking $\alpha(1\rightarrow4)$ linked (sulfated) L-fucosyl residues. Nevertheless, this enzymatic degradation of the *S. mcclurei* fucoidan is a novel finding as enzymatic modification of the *S. mcclurei* fucoidan has not previously been reported. The apparent lack of action of FcnA2 on the fucoidan from *T. ornata*, *S. cichorioides*, and *U. pinnatifida* suggests that FcnA2 does not catalyse cleavage of $\alpha(1\rightarrow3)$ bonds between fucosyl residues whereas the activity on the other three substrates supports that the enzyme attacks $\alpha(1\rightarrow4)$ bonds between L-fucosyl residues as previously shown²⁴.

Fda2 catalyses cleavage of $\alpha(1\rightarrow3)$ fucosyl bonds in sulfated fucoidan backbones

Fda2 also catalysed partial degradation of the galactofucan-rich fucoidan from *S. mcclurei* (Fig. 2C). The C-PAGE results also showed that this enzyme exerted weak and almost equal extents of degradation of the fucoidans from *F. vesiculosus* and *F. evanescens*, and very low activity on the fucoidans rich in $\alpha(1\rightarrow3)$ fucosyl linkages from *T. ornata*, *S. cichorioides*, and *U. pinnatifida* - the activity was very low, but still visible on the *S. cichorioides* fucoidan (with a smear at the top of the gel and weak bands in the lower part of the gel) and on the *U. pinnatifida* fucoidan (with a discernible smear at the top of the gel) (Fig. 2C).

The action of Fda2 on *S. mcclurei* fucoidan is a new finding that shows promise for employing the Fda2 enzyme for controlled degradation of the complex galacto-fucan fucoidan from *S. mcclurei*. As mentioned, fucoidans from *F. evanescens* and *F. vesiculosus* contain alternating $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ glycosidic bonds between the α -L fucosyl residues in their backbone structures. Hence the activity of the enzyme on *S. mcclurei*, *F. evanescens* and *F. vesiculosus* together with the weak activity on the substrates rich in $\alpha(1\rightarrow3)$ fucosyl linkages corroborated previous claims of the action of Fda2 on $\alpha(1\rightarrow3)$ bonded L-fucosyls in fucoidan²⁵. Both Fda1 and Fda2 were previously shown to digest sulfated fucans from *K. crassifolia* (i.e. *S. sculpera*) with the backbone structure $[3)\text{-}\alpha\text{-L-Fucp-(2OSO}_3\text{)-1}\rightarrow3\text{-}\alpha\text{-L-Fucp-(2,4OSO}_3\text{)-(1}\rightarrow\text{)]}$ and to partially digest fucoidan from other brown algae of the order Laminariales, such as *Saccharina japonica*, *Lessonia nigrescens*, and *Ecklonia maxima*³⁵. Hence, the data obtained support that Fda2, despite its instability (Fig. 1B), catalyses cleavage of $\alpha(1\rightarrow3)$ fucosyl bonds in sulfated fucoidan backbones.

FdIA and FdIB action

The FdIA and FdIB enzymes originating from *Flavobacterium* sp. SA-0081 (previously referred to as “*Fucobacter marina*”) (Table 1) were previously claimed to be specific for certain sulfated fuco-glucuronomannan (SFGM) structural fragments containing uronic acid and D-mannosyl α -linkages in fucoidan molecules²⁵. The enzymes were purified from SA-0082 strain and were shown to catalyse cleavage of SFGM fractions purified from the brown algae *Kjellmaniella crassifolia* (now *S. sculpera*) and to do so via a lyase mechanism cleaving the α -linkage between D-mannosyl and D-glucuronate in the SFGM fractions³⁶.

In this study FdIA and FdIB both exerted activity on the fucoidans from *S. mcclurei*, *F. vesiculosus*, and *F. evanescens*, only weak action on fucoidans from *T. ornata*, and essentially no activity on *S. cichorioides*, and *U. pinnatifida* (Fig. 2D and E). Fucoidan preparations from *S. mcclurei*, *T. ornata*, *F. evanescens* and *F. vesiculosus* may contain very low amounts of uronic acid and sometimes traces of mannose^{12, 14, 37, 38} but until now no data show that D-mannosyl and D-glucuronate are present in the backbone of these fucoidans. Moreover, no lyase activity was detected by monitoring absorbance at 232 nm indicating that the degradation products did not include unsaturated uronic oligosaccharides. FdIA and FdIB most likely cleave $\alpha(1\rightarrow4)$ fucosyl bonds in the backbone of these fucoidans, since the lack of activity on fucoidan from *S. cichorioides* and from *U. pinnatifida* (and the weak action on *T. ornata* fucoidan) indicated that FdIA and FdIB do not cleave $\alpha(1\rightarrow3)$ bonds in fucoidan.

The similar weak extent of degradation of the *F. vesiculosus* and *F. evanescens* fucoidan by both enzymes, producing almost similar oligomer profiles in the C-PAGE, suggests preference for rare or complex (e.g. C2 and C4) fucosyl-sulfatation in the *Fucus* fucoidan substrates – maybe of a type which occurs more abundantly in the *S. mcclurei* fucoidan, and most likely attacking only $\alpha(1\rightarrow4)$ fucosyl-bonds. The FdIB appeared to exert a more profound action on the *F. evanescens* substrate than the FdIA. Interestingly, the action of the two enzymes on *S. mcclurei* galactofucan substrate produced a band which travelled further in the gel than the sulfated tetra-saccharide of the control, suggesting that both FdIA and FdIB are able to catalyse disintegration of sulfated fucoidan oligomers. Due to the high degree of depolymerisation, down to oligosaccharides of less than DP4 (Fig. 2) and due to the high abundance of galactosyl residues in *S. mcclurei* fucoidan, we cannot rule out that FdIA and FdIB may cleave galactosyl- $\alpha(1\rightarrow4)$ bonds (Fig. 2D, E), although this needs further analysis.

Further assessment of *Sargassum mcclurei* fucoidan degradation

C-PAGE and SEC of oligosaccharides released by FcnA2, Fda2, FdIB and FdIA after extended reaction for 48 hours showed that each enzyme catalysed profound degradation of *S. mcclurei* fucoidan (Fig. 3). In all cases, the smallest oligosaccharide ran further than the lowest of the standard, suggesting that the released oligosaccharides are either smaller or more charged, i.e. more sulfated, than the tetra-saccharide in the standard. The SEC profiles of the FcnA2 and Fda2 were similar, but the product profile differed from those of FdIA and FdIB as there was a smaller peak at ~22.5 min. (corresponding to a molecular weight SEC standard of ~1.3 kDa). In contrast, FdIA and FdIB both produced a peak in the SEC around 22.5 min, indicating that they acted slower if at all on certain fucoidan fragments ≤ 1.3 kD. Taken together with the C-PAGE results (Fig. 3A), these data suggest that FdIA and FdIB exerted similar substrate attack preferences and did leave behind some oligomers around 1.3 kDa, whereas FcnA2 and Fda2 appeared to degrade the lower molar weight oligomers to a more significant extent.

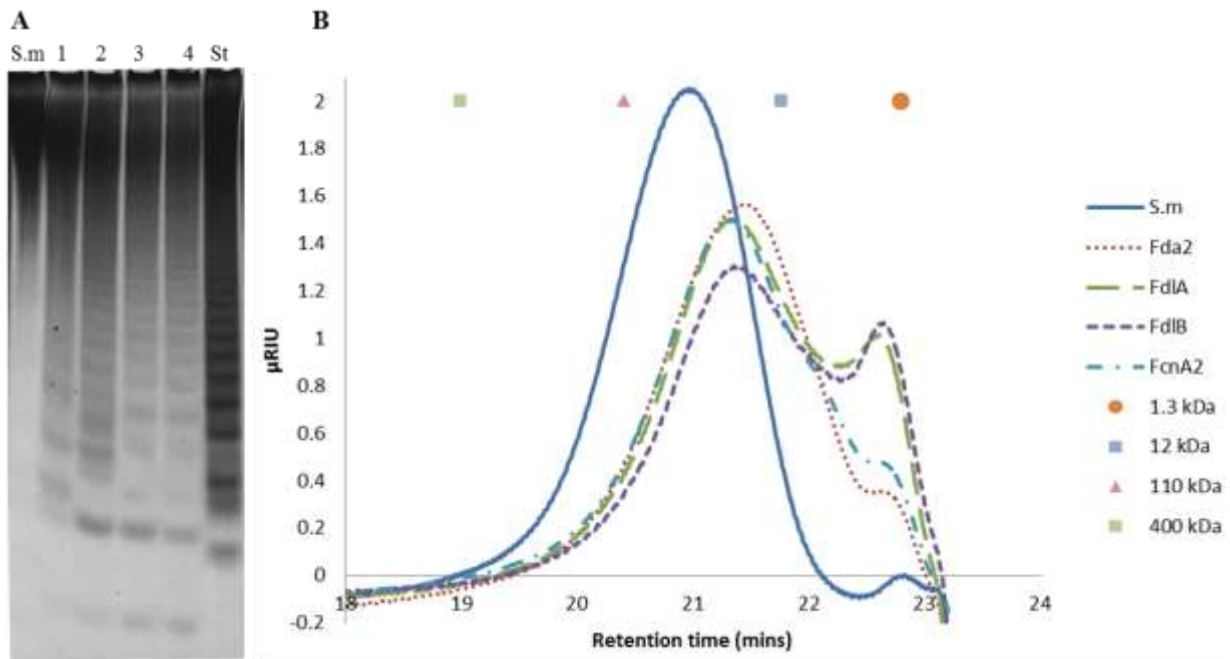


Figure 3: *Sargassum mcclurei* fucoidan (S.m) degraded by fucoidanases. A: C-PAGE and B: Size exclusion chromatography (SEC) of 1) FcnA2, 2) Fda2, 3) FdlA, 4) FdlB on *S. mcclurei* fucoidan and molecular weight standards. Reaction time was 48 hours. St in the C-PAGE is a hydrolysis standard from FFA2 treatment of fucoidan from *F. evanescens*²⁷.

New construct of FcnA2

Western blot analysis of FcnA2 (Fig. 1B) indicated that the spontaneous degradation of FcnA2 occurred from the C-terminal end, since the N-terminal His-tag was still present, making the protein visible in the western blot. To avoid this degradation a further truncation was made in FcnA2 by removing additional 80 amino acids from C-terminal end. This truncated enzyme was thus 229 amino acids shorter than the original FcnA enzyme and was called FcnAΔ229 (Table 1). FcnAΔ229 could be expressed very well and purified to high degree as illustrated by SDS-PAGE and western blot analysis giving the expected band size of 80 kDa with no apparent protein degradation (Fig. 4A, B).

FcnAΔ229 recombinant enzyme showed activity on the same substrates as FcnA2 (Fig. 4C). However, when degrading fucoidan from *S. mcclurei* an oligosaccharide was released after 24 hours that was running further than what was observed for FcnA2 (Fig. 4C). This result indicated that the change in stability conferred by deletion of the 80 amino acids in FcnA2 in turn apparently enhanced substrate degradation, but the truncation did not confer any other apparent changes in the *S. mcclurei* degradation profile.

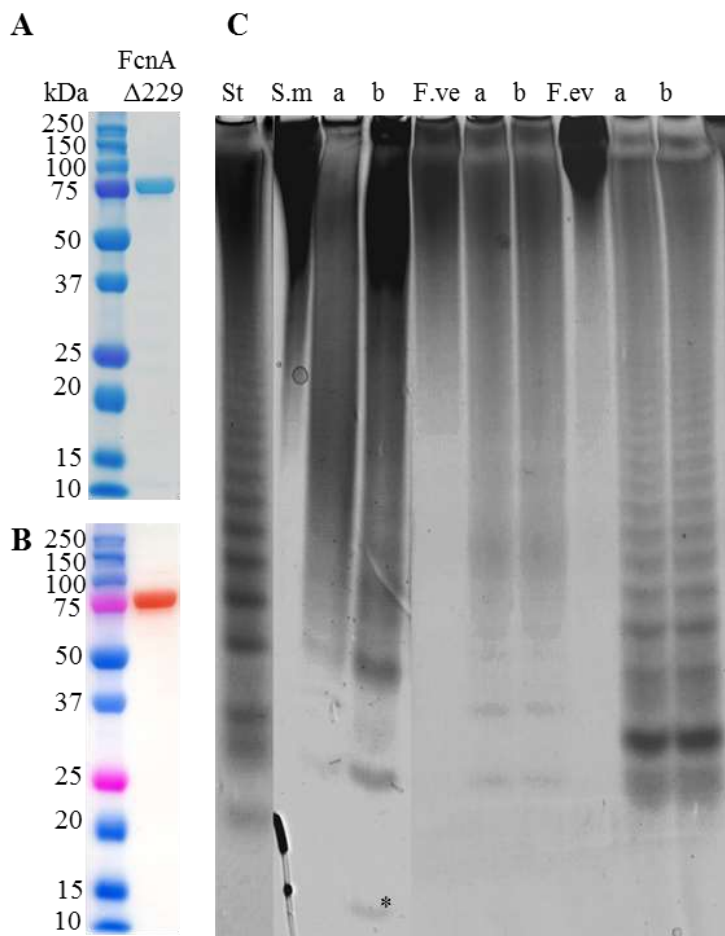


Figure 4. Purification and enzyme activity of FcnAΔ229. A) SDS-PAGE indicating the expected molecular weight of 80 kDa and very pure protein, B) Western blot of purified FcnAΔ229. (St) is the protein plus molecular weight marker, C) Enzyme activity by C-PAGE of a) FcnA2 and b) FcnAΔ229 on fucoidans from *S. mcclurei*, *F. vesiculosus*, and *F. evanescens*. FcnA2 and FcnAΔ229 have similar profiles on *F. vesiculosus* and *F. evanescens* fucoidans. The reaction time was 24 hours. *FcnAΔ229 produced an oligosaccharide of lower molecular weight than FcnA2.

Stabilization through C-terminal truncation of Fda1 and Fda2

The expression of Fda1 was high but the protein was retained in the cell debris after sonication (Supplementary Fig. S1). By analysis of the sequence of Fda1 and Fda2 it was found that both enzymes contained two predicted Laminin G domains (IPR001791) (LamG domains) towards the C-terminal (Supplementary Fig. S2). Moreover, because western blot analysis of Fda2 (Fig. 1B) indicated that enzyme destabilization occurred via degradation from the C-terminal as observed for FcnA2. A strategy to stabilize the enzymes was carried out involving deletion of the two predicted LamG domains in Fda1 and in Fda2. Hence, new constructs of Fda1, called Fda1Δ145 (one LamG domain deleted) and Fda1Δ395 (both LamG domains deleted) were prepared, each with an additional his-tag (Table 1). The additional his-tag was included as a new strategy in the construct to ensure better binding to the Ni²⁺ Sepharose column. Since Fda2, in addition to being highly unstable, substantial amounts of protein were lost during purification, presumably due to lack of binding to the column (data not shown) and to both stabilize the enzyme and ensure better binding to the column during purification a construct was made with an additional C-terminal his-tag. This new construct was called Fda2-His (Table 1). In addition, as for Fda1, new constructs devoid of either one or both of the two predicted LamG domains of Fda2 were also constructed. These Fda2 C-terminal deletion mutants were named Fda2Δ146 and Fda2Δ390 (Table 1, Supplementary Fig. S2).

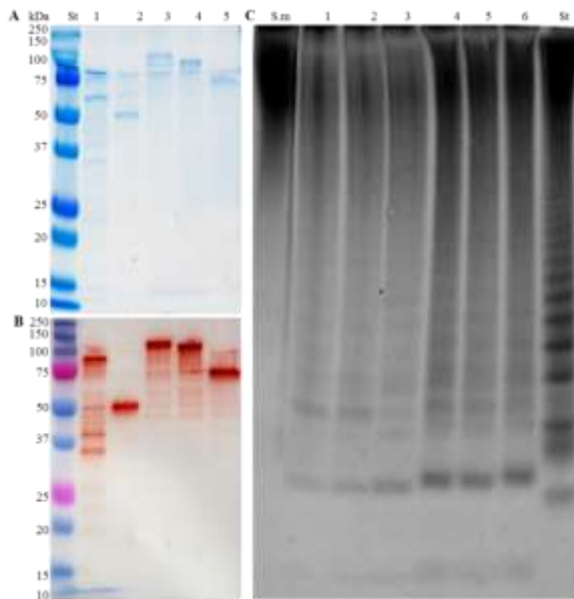


Figure 5. Purification and activity of Fda1 and Fda2 deletion mutants. A) SDS-PAGE and B) western blot of purified 1) Fda1 Δ 145, 2) Fda1 Δ 395, 3) Fda2-C-His, 4) Fda2 Δ 146, and 5) Fda2 Δ 390. (St) is the protein plus molecular weight marker. The expected sizes of the proteins were 90, 50, 125, 110, 70 kDa respectively. C) Enzymatic *S. mcclurei* fucoidan (S.m) degradation by C-PAGE: 1) Fda1 Δ 145, 2) Fda1 Δ 395; 3) Fda2-His; 4) Fda2 Δ 146; 5) Fda2 Δ 390; 6) Fda2 and the standard (St) resulting from FFA2 treatment of fucoidan from *F. evanescens*. The reaction time was 48 hours.

SDS-PAGE and Western blot analysis showed that all enzymes were expressed well. Some protein degradation was evident, but notably the double LamG deletion constructs, Fda1 Δ 395 and Fda2 Δ 390, appeared more stable than the less truncated enzymes (Fig. 5A, B). All the truncated enzymes exerted activity on *S. mcclurei* fucoidan, verifying the enzyme stabilization strategy by LamG deletion (Fig. 5C). Further study verified that Fda1 Δ 395 was stable, but that degradation of the other truncated enzymes (Fda1 Δ 145, Fda2-C-His, Fda2 Δ 146, and Fda2 Δ 390) occurred already inside the *E. coli* cells, since degradation was evident in the *E. coli* cells before sonication (Supplementary Fig. S3).

C-terminally truncated Fda1 attacks $\alpha(1\rightarrow3)$ -linkages

The truncated Fda1 proteins Fda1 Δ 145 and Fda1 Δ 395 both catalysed the degradation of most of the fucoidan substrates analysed in this study (Fig. 6). Furthermore, both truncated enzymes showed comparable degradation patterns, releasing fucoidan oligosaccharides that migrated equally in C-PAGE. Interestingly, Fda1 mutants were able to catalyse the degradation of fucoidans rich in $\alpha(1\rightarrow3)$ fucosyl linkages from *T. ornata*, *S. cichorioides*, and *U. pinnatifida* (Fig. 6), indicating that the C-terminally truncated Fda1 enzymes attack $\alpha(1\rightarrow3)$ -linkages as previously described²⁵. Removal of up to 47% of the Fda1 sequence from the C-terminal results in a more stable and still active enzyme, indicating that the 47% of the enzyme is not important for enzyme activity.

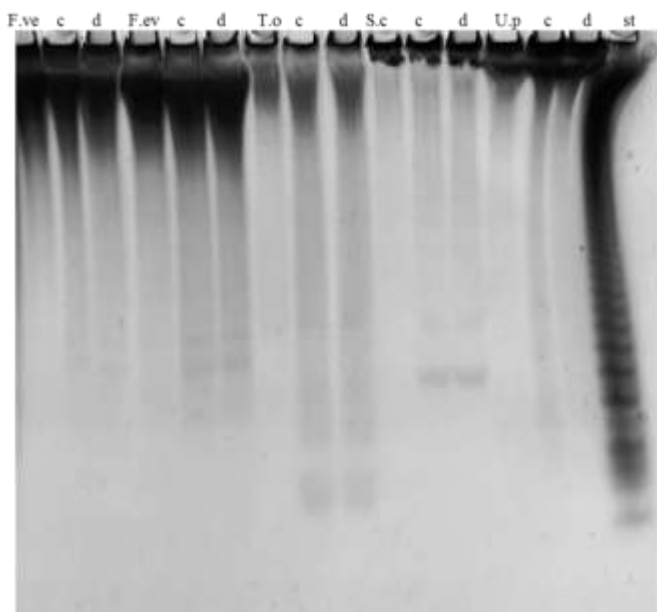


Figure 6. Enzyme activity of truncated Fda1 mutants by C-PAGE. Enzyme activity of c) Fda1 Δ 145 and d) Fda1 Δ 395 on fucoidans from *F. vesiculosus* (F.ve), *F. evanescens* (F.ev), *T. ornate* (T.o), *S. cichorioides* (S.c) and *U. pinnatifida* (U.p), standard (st). Both enzymes show activity on all the tested substrates to a comparable degree.

The work showed that fucoidan-degrading enzymes can exert activity on an array of different fucoidan substrates from brown macroalgae, even the very complex *S. mcclurei* fucoidan. FcnA2, Fda2, FdlA, and FdlB were thus all found able to degrade *S. mcclurei* fucoidan, with Fda2, FdlA, and FdlB having particularly high activity on this fucoidan known to contain sulfated galacto-fucan structural units and both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow3)$ L-fucosyl linkages. FcnA2 and FcnA2 Δ 229 were more active than all the other enzymes on *F. evanescens* fucoidan than on *F. vesiculosus* suggesting an effect on the enzymatic action of the substrate sulfatation pattern (and perhaps other effects). Fda2 was the only enzyme exerting degradation of the fucoidans rich in $\alpha(1\rightarrow3)$ L-fucosyl linkages, but FdlA and FdlB were also able to partially degrade the fucoidan from *T. ornata*. FdlA and FdlB, previously claimed to be lyases acting on manno-glucurono-linkages in fucoidan from *K. crassifolia* (i.e. *S. sculpera*), were specifically found to act as endo-fucoidanases on fucoidans devoid of these types of bonds (and to not produce any unsaturated 4-5 oligosaccharide uronides).

Stabilization of the enzymes was successfully achieved by double his-tagging and notably by targeted truncation of the C-terminal ends in FcnA2, Fda1 and Fda2. Interestingly, for FcnA2 the stabilization by C-terminal truncation produced an FcnA2 variant, called FcnA Δ 229, which appeared able to foster more profound degradation of the *S. mcclurei* fucoidan than the parent enzyme. For Fda1 and Fda2 successful expression and stabilization was attained by LamG domain deletion, in turn this stabilization allowed us to show the ability of the otherwise unstable Fda1 to catalyse degradation of the *S. mcclurei* fucoidan. The data obtained have implications for use of these enzymes, including the stabilized versions, in fucoidan processing. Hopefully, in turn allowing manufacturing of homogenous bioactive fucoidan oligomer products.

Materials and methods

Fucoidan substrates

Crude fucoidans from *Sargassum mcclurei*, *Fucus evanescens*, *Undaria pinnatifida*, and *Saccharina cichorioides* were extracted as described by Zvyagintseva et al. (1999)³¹. Fucoidan from *S. mcclurei* was purified further by ion-exchange chromatography¹². *Turbinaria ornate* fucoidan was extracted as described by Thanh et al. (2013)¹³. Fucoidans from *F. evanescens*, *U. pinnatifida*, and *S. cichorioides* were purified as described by Kusaykin et al. (2006)³². Pure fucoidan from *Fucus vesiculosus* (F8190) was purchased from Sigma-Aldrich (Steinheim, Germany).

Enzymes and gene constructs

Amino acid sequences for the five enzymes FcnA2, Fda1, Fda2, FdlA, and FdlB were retrieved from GenBank (Table 1). The construct containing the gene encoding FcnA2 was designed to harbour an N-terminal 6xhistidine tag, while the gene constructs *Fda1*, *Fda2*, *FdlA*, and *FdlB* encoding the Fda1, Fda2, FdlA, FdlB proteins, respectively, were designed to harbour an N-terminal 10xhistidine tag. The synthetic codon-optimized genes (optimized for *E. coli* expression), all devoid of their original signal peptide, were synthesized by GenScript (Piscataway, NJ, USA) and delivered as inserted either into the pET-45b(+) vector between the KpnI and PacI restriction sites (FcnA2) or into the pET-19b(+) plasmid vector between the NcoI and XhoI restriction sites (all other enzymes).

For FcnA2 C-terminal deletion of 80 amino acids of the enzyme equivalent to deletion of 229 amino acids of FcnA (GenBank no. CAI47003.1) was constructed, and the truncated protein was named FcnAΔ229 (Table 1).

Both Fda1 and Fda2 contain 2 predicted laminin G (LamG) domains in the sequence. Deletion mutants devoid of one or both predicted LamG domains were constructed by PCR amplification of the codon-optimized genes, each with an additional C-terminal 10xhistidine tag, using CloneAmp HiFi polymerase premix (Takara Bio USA Inc., Mountain View, CA, USA) (primer sequences are listed in the Supplemental Material Table S1). For Fda1, the truncated proteins were named Fda1Δ145 and Fda1Δ395, as 145 and 395 amino acids had been removed from the C-terminal end, respectively. Analogously, for Fda2, the truncated versions were named Fda2Δ146 and Fda2Δ390, indicating that 146 and 390 amino acids, respectively, had been removed from the C-terminal. The construct of Fda2-His was done by adding 10xhistidine tag at the C-terminal end. After PCR amplification, products were digested with BsaI and XhoI and ligated into the pET19b (+) vector between the NcoI and XhoI sites. Positive clones were confirmed by DNA sequencing.

The *Escherichia coli* strain DH5α (Invitrogen® Life Technologies, Thermo Fisher Scientific, MA, USA), was used as plasmid propagation host. BL21 (DE3) and C41 (DE3) (also from Invitrogen® Life Technologies) were used as expression hosts for the fucoidan-degrading enzymes (Table 1). Protein expression was done as described below.

Table 1. Fucoidan-degrading enzymes, features, molecular weight, and expression strains used.

Enzyme name/ GenBank no.	Organism	Features ^a	Length (aa) ^b	Expected mol. weight (kDa)	<i>E. coli</i> expression strain
FcnA CAI47003.1	<i>Mariniflexile</i> <i>fucanivorans</i> SW5	nd	1007	nd	Nd
FcnA2	<i>Mariniflexile</i> <i>fucanivorans</i> SW5	His ₆ (N-term)	799	88	BL21 (DE3) pGro7 ^c

FcnAΔ229	<i>Mariniflexile fucanivorans</i> SW5	His ₁₀ (N-term)	720	80	BL21 (DE3) pGro7 ^c
Fda1	<i>Alteromonas</i> sp.	His ₁₀ (N-term)	804	87	BL21 (DE3) pGro7 ^c
AAO00508.1	SN-1009				
Fda1Δ145	<i>Alteromonas</i> sp.	His ₁₀ (N-term) and His ₁₀ (C-term)	669	73	BL21 (DE3) pGro7 ^c
	SN-1009				
Fda1Δ395	<i>Alteromonas</i> sp.	His ₁₀ (N-term) and His ₁₀ (C-term)	419	46	BL21 (DE3) pGro7 ^c
	SN-1009				
Fda2	<i>Alteromonas</i> sp.	His ₁₀ (N-term)	868	94	BL21 (DE3) pGro7 ^c
AAO00509.1	SN-1009				
Fda2-His	<i>Alteromonas</i> sp.	His ₁₀ (N-term) and His ₁₀ (C-term)	878	95	BL21 (DE3) pGro7 ^c
	SN-1009				
Fda2Δ146	<i>Alteromonas</i> sp.	His ₁₀ (N-term) and His ₁₀ (C-term)	732	80	BL21 (DE3) pGro7 ^c
	SN-1009				
Fda2Δ390	<i>Alteromonas</i> sp.	His ₁₀ (N-term) and His ₁₀ (C-term)	488	53	BL21 (DE3) pGro7 ^c
	SN-1009				
FdlA	<i>Flavobacterium</i> sp.	His ₁₀ (N-term)	684	74	C41 (DE3)
AAO00510.1	SA-0082				
FdlB	<i>Flavobacterium</i> sp.	His ₁₀ (N-term)	692	76	C41 (DE3)
AAO00511.1	SA-0082				

nd: not determined in this study.

^a Wild type signal peptide had been removed for codon-optimized synthesized construct.

^b Including the his-tags

^c groES-groEL chaperone expressed from the pGro7 plasmid

Production of recombinant enzymes

Expression of FcnA2 and FcnAΔ229 was performed in *E. coli* (BL21 (DE3) harbouring the Pch2 (pGro7) plasmid. Overnight cultures grown at 37°C with agitation (180 rpm) in lysogeny broth (LB) medium containing 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol were used to inoculate 500ml LB containing 100µg ml⁻¹ ampicillin, 34µg ml⁻¹ chloramphenicol and 0.05% arabinose. The inoculated LB was incubated at 37°C with 180 rpm shaking until OD₆₀₀ = 0.6-0.8. Enzyme expression was induced with 1mM IPTG for 20 hours at 20°C and 180 rpm.

Expression of Fda1, Fda1Δ145, Fda1Δ395, Fda2, Fda2-His, Fda2Δ146, Fda2Δ390 was also performed in *E. coli* (BL21 (DE3) with Pch2 (pGro7)). Overnight cultures grown at 37°C and 180 rpm in LB medium containing 100µg ml⁻¹ ampicillin and 34µg ml⁻¹ chloramphenicol were used to inoculate 500ml auto-induction media containing 0.6% Na₂HPO₄, 0.3% KH₂PO₄, 2%, tryptone, 5% yeast extract, 5% NaCl, 0.6% glycerol, 0.05% glucose, 0.2% lactose, 0.05% arabinose, 100µg ml⁻¹ ampicillin and 34µg ml⁻¹ chloramphenicol. Cells were grown at 20°C and 180 rpm for 20 hours.

Expression of FdlA, FdlB was performed in *E. coli* (C41 (DE3)). Overnight cultures grown at 37°C and 180 rpm in LB medium containing 100 µg ml⁻¹ ampicillin were used to inoculate 500ml LB containing 100µg ml⁻¹ ampicillin and

were grown at 37°C and 180 rpm until $OD_{600} = 0.6-0.8$. The expression of the recombinant fucoidanases was induced with 1 mM IPTG during cell growth for 20 hours at 20°C and 180 rpm.

Cells were harvested by centrifugation at 5000xg for 20min and 4°C and the pellet was re-suspended in binding buffer (20 mM Tris-HCl buffer, 250 mM NaCl, 20 mM imidazole, pH 7.5) before being disrupted by UP 400S Ultrasonic processor with 0.5 cycle and 100% amplitude. Cell debris was pelleted by centrifugation (20,000xg, 20 min at 4°C). The supernatant obtained by centrifugation was then filtered through a 0.45µm filter and applied to a 5ml Ni^{2+} Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden) which was equilibrated with binding buffer using an Äkta purifier (GE Healthcare, Uppsala, Sweden). The resin was washed 3 times with 20mM Tris-HCl buffer, 250 mM NaCl, 20 mM imidazole, pH 7.5 and proteins were eluted by a linear gradient of elution buffer (20 mM Tris-HCl buffer, 250 mM NaCl, 20 mM to 500mM imidazole, pH 7.5). The eluted fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described below to assess the purity, and homogenous fractions were pooled. Protein content was determined by the Bradford assay³³ with bovine serum albumin as standard.

SDS-PAGE

The homogeneity and molecular weight of the recombinantly expressed proteins were estimated by (SDS–PAGE) electrophoresis according to the Laemmli protocol³⁴. Electrophoresis was performed in 12% acrylamide gels with the addition of 4xlaemmli loading-buffer, to 40µg of crude protein and 5µg purified protein and 5mM DTT. The analysis of total intracellular proteins was conducted by using the biomass from 300µl culture with 100µl of 4xLaemmli loading-buffer, 10µl of samples were loaded on the 12% acrylamide gels. The Protein Plus molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA) with molecular weights of 10–250 kDa was used as standard.

Western blot analysis of proteins

Total intracellular protein, crude enzymes (40µg) and pure enzymes (5µg) were separated by 12% acrylamide gels with the addition of 4xlaemmli loading-buffer. Separated proteins were transferred onto a PVDF blotting membrane (GE Healthcare No. 1060022) and blotted in Tris-glycine pH 8.3 running buffer at 100V for 45 min, after activation of the membrane in 96% ethanol for around 10 sec. The membrane was blocked with 2% skim milk in 0.01M TBS (Tris-based sodium chloride pH 7.6) buffer containing 0.1% Tween 20 (TBS_T buffer) for 60 min. The membrane was then incubated in TBS_T buffer with monoclonal anti-polyhistidine peroxidase conjugated antibody (Sigma-Aldrich, Steinheim, Germany) at 1:10.000 dilutions in a total volume of 30ml for 1 hour. The membrane was washed in TBS_T buffer 3x10 min and TBS with 0.1% Tween20 for 20 mins. The bound antibodies were detected by horse radish peroxidase using the AEC Kit (Sigma-Aldrich, Steinheim, Germany) according to manufacturer's protocol.

Carbohydrate–Polyacrylamide Gel Electrophoresis (C-PAGE)

Reaction mixtures containing 0.5µg/µl enzyme solution in 20mM Tris–HCl buffer pH 7.4, 250mM NaCl and 10mM $CaCl_2$ (buffer A) and 1% weight/volume fucoidan in buffer A were incubated at 35°C for 24h–48h. Each reaction mixture (10µl) was mixed with 5µL loading buffer (20% glycerol and 0.02% phenol red). Samples (5µL) were electrophoresed at 400V through a 20% (w/v) 1 mm thick resolving polyacrylamide gel with 100mM Tris-borate buffer pH 8.3 for 1h. Gel staining was performed in two steps, first with a solution containing 0.05% alcian blue 8GX (Panreac, Spain) in 2% acetic acid for 45 mins and then with 0.01% O-toluidine blue (Sigma-Aldrich, Steinheim,

Germany) in 50% aqueous ethanol and 1% acid acetic. The hydrolysate standard was a hydrolysate obtained after enzymatic reaction of FFA2 on *Fucus evanescens* fucoidan²⁷.

SEC analysis

High Performance Size Exclusion Chromatography was performed using an Ultimate iso-3100 SD pump with a WPS-3000 sampler (Dionex) connected to an RI-101 refractive index detector (Shodex). 100 μ L of 3 times diluted reaction mixtures was loaded on a Shodex SB-806 HQ GPC column (300 x 8 mm) equipped with a Shodex SB-G guard column (50 x 6 mm) (Showa Denko K.K. Tokyo, Japan). Elution was performed with 100 mM sodium acetate pH 6 at a flow rate of 0.5 mL/min at room temperature. Pullulan standards were used as references.

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Author contributions

H.T.T.C., M.D.M., M.J.L., and A.S.M. designed the research, and analysed and interpreted the data, and prepared the manuscript. A.S.S. and M.I.K. prepared the fucoidan from *F. evanescens*, *S. cichorioides* and *U. pinnatifida* and enzyme activity standard for C-PAGE. L.M.B., V.T.T.T., T.D.P. and B.H.T prepared the *S. mcclurei* and *T. ornata* fucoidans, and J.H. contributed the SEC analyses. All the authors have read and approved the final manuscript.

Competing interests statement

All authors declare no conflicts of interest.

Data availability

All data generated or analysed during this study are included in this article and its Supplementary Information file.

Additional information

There is a Supplementary information file accompanying this paper.